IDENTIFICATION OF CONSTITUTIVELY VERSUS INDUCIBLY EXPRESSED GENES IN THE STREPTOCOCCAL CONJUGATIVE TRANSPOSON Tn*5252*

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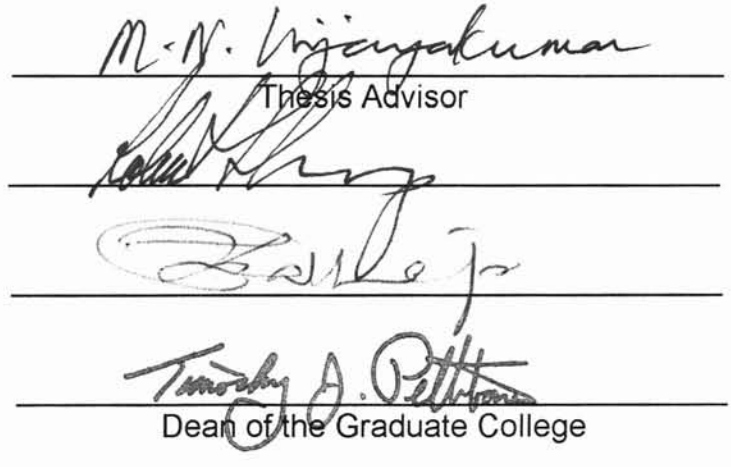

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CHAPTER I

INTRODUCTION

Multiple antibiotic resistances among clinical strains of *Streptococcus pneumoniae* have been of medical concern and biological interest since 1970s (16). Drug resistance among a number of clinical isolates of streptococci was reported to be associated with plasmids. However, there are numerous examples of drug-resistant clinical isolates that lack detectable plasmid DNA and several research groups were unable to show the association of multiple antibiotic resistances with plasmids (58,89). Previously, it has been shown that resistance characteristics in *S. pneumoniae* and beta hemolytic group A, B, F, and G streptococci can be transferred by a DNase-resistant process requiring cell-to-cell contact. (21,36). Later it was demonstrated that multiple-antibiotic resistance was associated with self-transferring genetic elements named conjugative transposons (7,15,26,31).

Conjugative transposons are mobile elements that can be transferred by a DNase-resistant process requiring cell-to-cell contact. Transposition of these elements can result in rearrangements of DNA sequences in the host genome. These elements have been discovered both in gram-positive and gram-negative bacteria. There are four major groups of conjugative elements: the *Bacteroides*

elements (62), the lactococcal elements (18), Tn*916*/Tn*1545* type streptococcal elements and Tn*5252*/*Tn3701* type complex streptococcal elements (77,37). Although these streptococcal elements are capable of conjugal transposition, they share no structural similarity (68).

Recently, novel conjugative transposon-like elements have been identified in *Vibrio cholerae* and *Salmonella senftenberg*. *V. cholerae*, a gram negative bacterium, causes lethal diarheal diseases cholera. A new serogroup of *V. cholera* O139 has multiple drug resistances to the antibiotics sulfamethozaxole, trimethoprim, streptomycin and furazolidone. It was found that the first three of these antibiotic resistances are carried on a 62 kb self transmissible, chromosomally integrating genetic element which is termed as SXT element. It is suggested that SXT element has a fairly broad host range including *E. coli* and *V. cholerae* O1. The SXT element integrates into the host chromosome by a site-specific mechanism that does not require *recA*. The properties of the SXT element are found to be similar to those of *Bacteroides* conjugative transposons (85). In *S. senftenberg* 5494-57 a 100 kb conjugative transposon, named CTn*scr94* have been identified. This genetic element is capable of self-transmission by conjugation and is able to integrate into target chromosome or plasmid by RecA-independent recombination process. CTn*scr94* codes for a sucrose fermentation pathway. It is a new example of a complex mobile genetic element being involved in fast and efficient horizontal gene transfer among enteric bacteria (30).

Transposons are capable of carrying drug resistance genes and considered to be the major cause of multiple antibiotic dissemination in clinical streptococci (57). In particular the emergence of multiple antibiotic resistance in streptococci and other related bacterial pathogens continues to be a major clinical concern.

In many ways conjugative transposons differ from other classes of transposons. These elements do not cause duplication of the target sequences when they integrate into the recipient genome. They share several characteristics with other gene transfer systems such as plasmids and phages (2). The mechanism involved in conjugative transposition is very similar to integration and excision of temperate bacteriophages such as bacteriophage lambda. It has been reported by several researchers that almost all conjugative transposons have a covalently closed circular intermediate in conjugation just like the circular intermediate in bacteriophage assembly.

Among these conjugative transposons the molecular biology of Tn*5253* has been analyzed in detail by Vijayakumar and coworkers (37,80,82). Tn*5253* is a 65.5 kb streptococcal composite conjugative element carrying genes encoding resistance to tetracycline and chloramphenicol. Tn*5253* was originally identified in *S. pneumoniae* BM6001 strain. The entire conjugative transposon was cloned in *E. coli* and a detailed restriction map of the element obtained in order to understand the molecular aspects of its structure and mechanism of transfer (2). After restriction fragment analysis, an 18 kb DNA fragment carrying a tetracycline resistance determinant was identified in the center of Tn*5253*. Upon removal

from the parental element (Tn*5253*), the smaller 18 kb element, termed as Tn*5251*, acted as an independent conjugative transposon. Tn*5251* displayed structural and functional similarities to Tn*916*/Tn*1545* family of conjugative transposons (80). In order to further investigative the characteristics of Tn*5253*, the 18 kb element (Tn*5251*) was deleted and a 47.5 kb element called Tn*5252*, which was also capable of conjugative transposition, was obtained (2). These results suggested that Tn*5253* was a composite structure of two independent conjugative transposons, Tn*5251* and Tn*5252*.

Further studies implicated that Tn*5252* shared the same conjugal transfer properties and target specificity with Tn*5253*. Thus, the efforts were focused on studying genetic and molecular organization of Tn*5252* to better understand the nature and evolution of the conjugative transposons. A functional map of Tn*5252* was determined by introducing deletions and insertions within the transposon (36). It was reported that, unlike Tn*916* elements, Tn*5252* inserts at a unique site in the chromosome by site-specific recombination (69). The studies showed that an 8 kb segment of Tn*5252* DNA flanked by IS-like elements carry a chloramphenicol resistance marker (*cat* gene). The *cat* gene was shown to be spontaneously cured as a result of recombination between two IS-like elements. The loss of *cat* gene did not affect conjugal transfer of Tn*5252*. Thus, it was concluded that the remaining 39 kb segment of Tn*5252* carried the essential genes for conjugal transfer. Using insertion mutagenesis strategy several workers were dedicated to identification of a total of 24 putative genes at the right and the left terminus of this element (82).

The putative DNA transfer related-genes were suggested to be clustered in the right terminal region of Tn*5252*. The DNA sequence near right terminus revealed the presence of the DNA transport genes in an operon like structure. In this region a cluster of at least 11 potential genes including a DNA cytosine-methyltransferase gene (ORF6) were discovered (64). The protein encoded by one of these genes, ORF21, was significantly similar to the group of proteins thought to facilitate the transmission of single stranded DNA across the cell membrane. Other genes like ORF26 and ORF28 were found to be related with the microtubule-binding protein and a hydrophobic membrane bound protein, respectively.

On the other hand, on about 6 kb of left terminal region of the transposon the presence of 13 potential genes were demonstrated. Among 13 open reading frames, ORF1 and 2 were identified as genes encoding a site-specific recombinase (83). ORF5 and ORF4 encode the excisionase and DNA relaxase, respectively (69). Computer analysis revealed four open reading frames ORF3, ORF5, ORF7 and ORF8 clustered in a 1.2 kb region. Later proteins of two genes ORF3 and ORF4 have been purified and characterized. Recently, the presence of two open reading frames, showing high level of similarities to *umuC* and *umuD* homologues involved in protection against UV irradiation, were demonstrated in a region located on the left terminus of Tn*5252*. Therefore, there is evidence that Tn*5252* has genes involved in SOS response (49).

In between the two clusters of transfer related genes at the termini of Tn*5252*, a 25 kb DNA segment devoid of genes related to conjugal mobility has

been identified. Conjugative transposons were isolated from a variety of clinical streptococcal species for several years. They were shown to carry regions highly similar to the central segment of Tn*5252* raising the possibility that proteins encoded by the genes of the central region may provide some type of selective advantage to the pathogenic bacteria within a host organism.

The aim of this study is to develop a genetic system to identify constitutively and inducibly expressed genes in Tn*5252* using Tn*917* and insertion-duplication mutagenesis.

CHAPTER II

LITERATURE REVIEW

Streptococci and *Streptococcus pneumoniae*

The streptococci are a heterogeneous group of gram-positive cocci that includes organisms commonly found among the flora of humans as well as organisms that cause both mild and severe diseases. Also, in this group are organisms that inhabit the environment outside the human body, organisms that cause disease in animals and organisms that are important in several industries such as dairy industry (67).

The streptococci are gram-positive, spherical cells that occur in chains of varying length. Most are facultative anaerobes whereas a few are obligate anaerobes. They are classified primarily by hemolytic behavior and antigenic characteristics associated with a cell wall carbohydrate called C substance (22).

Beta-hemolytic group A streptococci (*Streptococcus pyogenes*) are the most important human pathogens. *S. pyogenes* is involved in serious infections in humans, such as cellulites, pharyngitis, impetigo and acute rheumatic fever

(5). Group A streptococci can secrete a large number of proteins including some with the activity that suggests a role in pathogenesis. Antigens associated with pathogenesis include various toxins, hemolysins and spreading factors. The major antigen associated with pathogenesis is M protein, which inhibits phagocytosis along with capsule (6). M protein is considered as a possible anti-streptococcus vaccine.

Group B streptococci (*Streptococcus agalactiae*) and Viridans group including *Streptococcus gordonii*, *Streptococcus mutants* and *Streptococcus mitis* are inhabitants of oral cavity and are associated with dental caries and endocarditis (5, 22).

Among group D streptococci, *E. faecalis* is a part of normal flora in the intestinal tract. However, it is the major pathogen in the urinary tract infections. The anaerobic streptococci classified in the genera *Peptococcus* and *Peptosreptococcus* are found in the oral cavity and intestinal tract. They have been incriminated in such infections as subacute bacterial endocarditis and wound abscesses (5).

On the other hand, *S. pneumoniae,* which is also called pneumococcus, is an inhabitant of the upper respiratory tract and is the causative agent of otitis media and bacteremia in children. Also, it causes pneumonia and meningitis among all populations. The pneumococcus is a fastidious microorganisms that occurs singly, in pairs or in chains. Since it lacks the enzyme catalase, it requires addition of blood to the medium in order to proliferate. Pneumococcal colonies on blood agar are identified by alpha hemolysis around the colonies. Encapsulation

has been shown to be essential for virulence. However repeated subculturing in the laboratory leads to the loss of capsule making the laboratory strains less virulent (5,6,22).

S. pneumoniae is one of the model microorganisms that had been studied for several centuries by very well known scientists such as Louis Pasteur. Many important discoveries resulted from molecular genetic studies of *S. pnemunoniae*. The studies of Griffith and Avery demonstrating the ability of pneumococci to be transformed by exogenous DNA revealed that DNA is the genetic material (1). Other important discoveries resulting from the study of pneumococcus include the regulatory effects of competence factor, the role of capsule in avoidance of phagocytosis, the therapeutic effect of penicillin, and the identification of conjugative transposons including Tn*1545* and Tn*5253*.

Even today this microorganism is being studied intensively due to its involvement in several human and animal diseases.

Multiple Antibiotic Resistance in *Streptococcus pneumoniae*

Since the initial detection of plasmids in streptococci, it has become evident that the majority of the genes mediating resistance to antibiotics in this genus are plasmid borne. This type of resistance has spread in recent years among clinical isolates of all streptococcal species studied with the remarkable exception of *S. pneumoniae* (20).

In *S. pneumoniae*, multiple drug resistance has emerged since 1977 (34). Experiments conducted by Buu-Hoi and Horodniceanu showed that the resistance in pneumococci is a result of a novel form of conjugative transfer in the apparent absence of extrachromosomal DNA involvement (7,76). Additionally, DNase-resistant nature of antibiotic resistance dissemination eliminated the possibility of transfer by transformation. Today, the new groups of antibiotic resistance vehicles are known as "conjugative transposons".

The existence of conjugative transposons such as Tn*1545* in *S. pneumoniae* (20), Tn*3701* of *S. pyogenes* (39) and Tn*916* in *E. faecalis* (26) could clearly account for the spread of multiple drug resistance to and among pneumococci.

S. pneumoniae remains a major pathogen responsible for high morbidity and mortality in both developed and developing world. During the last few decades there has been a dramatic increase in the incidence of antibiotic resistant streptococci. In several countries 50-80% of pneumococcal strains isolated are penicillin resistant, which in most cases are resistant also to tetracycline, chloramphenicol, cotrimoxazole and erythromycin (19,42).

A plasmid-free clinical isolate of *S. agalactiae* B109 can transfer resistance to chloramphenicol, tetracycline and MLS antibiotics (macrolides, lincosamides) to group A, B, C, D, G and H streptococcal recipients, as well as pneumococcus. At least two conjugative resistance elements have been identified and genetically analyzed in these bacteria. One of the two elements is termed as *cat-erm-tet* element. It has been shown that *S. agalactiae* B109

transfers these markers by a conjugation-like process requires cell-to-cell contact (32).

Conjugative Transposons

Medically important gram-positive bacteria as well as gram-negative bacteria possess antibiotic resistance genes often determined by genes present in a class of transposable elements named conjugative transposons. These elements range in size from 18 kb to 100 kb. The gram-positive and gram-negative hosts harboring these elements act as conjugative donors and during a mating event the transposon is transferred to a new location in the genome of the recipient cell (68). Also conjugative transposons can excise and integrate elsewhere in the same cell. Conjugative transposition is a DNase-resistant and *recA* independent process (60).

Evidently, conjugative transposons seem to be significantly different from conventional transposons in that they have a circular intermediate transferred by conjugation and do not create target site duplication when they integrate (60,57,68).

Conjugative transposons are chimeric molecules. They combine features of transposons, plasmids, and bacteriophages. These elements are phage-like molecules such that their excision and integration resemble those of temperate bacteriophages and some have a circular intermediate. Interestingly, nucleotide sequence analysis of integrases of some conjugative transposons suggests that they are members of the lambda integrase family (60,72,54). Many conjugative

transposons can mobilize coresident plasmids, but unlike plasmids these elements are devoid of autonomous replication in the host (60). Conjugative transposons were first isolated from pathogenic streptococcal strains. Among the most studied conjugative transposons are: Tn*916* of *E. faecalis*; Tn*3071* identified in *S. pyogenes*; Tn*1545* and Tn*5253* isolated from *S. pneumoniae*. All conjugative transposons isolated to date carry *tetM* gene or a closely related gene for tetracycline resistance (68).

Conjugative Transposons of Gram Negative Bacteria

Non-replicating *Bacteroides* units (NBUs) are 10-12 kb transfer defective forms of conjugative transposons identified in *Bacteroides*, a group of gram-negative anaerobic microorganisms (61). Possibly, NBUs have covalently closed circular intermediate just like conjugative transposons. These elements are excised and mobilized by a conjugative transposon in which case excision and integration resembles those of phage lambda (51). Also, SXT element from *V. cholerae* O139 and CTnscr94 of *S. senftenberg* 5494-57 are recently identified conjugative transposons in gram-negative bacteria (85,30).

Conjugative Transposons of Gram Positive Bacteria

Tn*5276* Element: The 70 kb transposon Tn*5276*, originally identified in *Lactococcus lactis* (55), was shown to be conjugally transferred to other *L. lactis*

strains. This element carries the genes for nisin production and sucrose fermentation, but it does not carry any antibiotic resistance genes (56).

Tn*916*-Tn*1545* Family: Members of the Tn*916*-Tn*1545* family of conjugative transposons are ubiquitous among gram-positive bacteria. Their presence in more than 50 different bacterial species, both gram-positive and gram-negative, implies that they can escape restriction mechanisms within a broad host range. As well as conjugative transposition these elements can transpose intracellularly to other mobile DNA molecules such as plasmids and other conjugative transposons. The elements in this family do not show any target specificity for insertion (14).

The size of the elements in this family range from 18 kb to 25 kb. They carry the widely disseminated tetracycline resistance gene *tetM* and some members of this family can carry determinants for erythromycin and kanamycin resistance (18).

Tn*916*, an 18 kb element, was the first conjugative transposon to be identified. It was discovered on the chromosome of the hemolytic multi-drug resistant *E. faecalis* DS16 (72).

Tn*1545* is a 25.3 kb element originally identified in *S. pneumoniae* BM4200. This element harbors resistant determinants for tetracycline erythromycin and kanamycin. Interestingly, Tn*1545* share the same transposition mechanism with Tn*916* (18,8).

Tn*3071*-Tn*5253* Family: This is a group of complex conjugative transposons that are reported to be 50 kb or more in size. Each of these elements carries a region very similar to Tn*916*. Additionally they harbor many antibiotic resistant determinants.

Tn*3701*, a 50 kb element, was originally identified in *S. pyogenes* A54. This element encodes for chloramphenicol, erythromycin and tetracycline resistance genes (39).

Tn*5253*, a 65.5 kb conjugative transposon, was first identified in *S. pneumoniae* BM6001 (74). This conjugative transposon encoding chloramphenicol and tetracycline resistance genes was initially called Ω *cat tet* element. This element is shown to insert into a preferred target region of wild type *S.pneumoniae* Rx1 chromosome (80). Tn*5253* was studied in detail by Vijayakumar, et al., using insertion mutagenesis by inserting *E. coli* plasmid vector pVA891 at several sites within the transposon (47). After the recovery of the fragment of Tn*5253* on pVA891 derivative plasmids, a restriction map of was constructed; resistance genes and target sites of the transposon were identified. Further experiments conducted by Ayoubi et al. showed that Tn*5253* is a composite structure of two conjugative transposons, Tn*5251* and Tn*5252* (2). An 18 kb *tet*-carrying element, Tn*5251*, was inserted within a 47.5 kb transposon Tn*5252*. LeBouguenec et al. found that Tn*5251* was homologous to Tn*916* after hybridization studies and restriction mapping (39). Tn*5251* functional similarity to Tn*916* was demonstrated by Ayoubi et al. (2). Also, it was shown that Tn*5251* and Tn*5252* didn't share any significant homology. Furthermore, Tn*5251* was

reported to transpose into different targets on *S. pneumoniae* genome. Tn*5252* was further studied in detail by Vijayakumar, et al. (Fig. 1).

Tn5252

Tn*5252* is a conjugative transposon made up of the sequences beyond Tn*5251* within Tn*5253*. This element was shown to transpose into a preferred target site on the pneumococcal chromosome (83). DNA sequence analysis of the target on the chromosome and terminal regions of the transposon showed a possible *att* site on the chromosome (83). A 72 bp DNA segment of the target region on the chromosome, *att*B, was identified at both ends of the transposon. The size of the att sites flanking the transposon, *att*L and *att*R, were almost the same as *att*B and these flanking copies also contained minor sequence differences. Interestingly, there were no minor sequence differences within the termini of Tn*5252*. On the other hand, *att*L was shown to be on Tn*5252* whereas *att*R was on the host chromosome. This observation suggested that Tn*5252* integrated as a result of a conservation recombination mechanisms very similar to site-specific insertion of phages (83).

Insertion mutagenesis using pVA891 led to identification of several clones carrying different fragments of Tn*5252*. Analysis of these clones resulted in identification of transfer-related regions at the right terminus and the genes involved in regulation and DNA-processing at the left terminus of Tn*5252*.

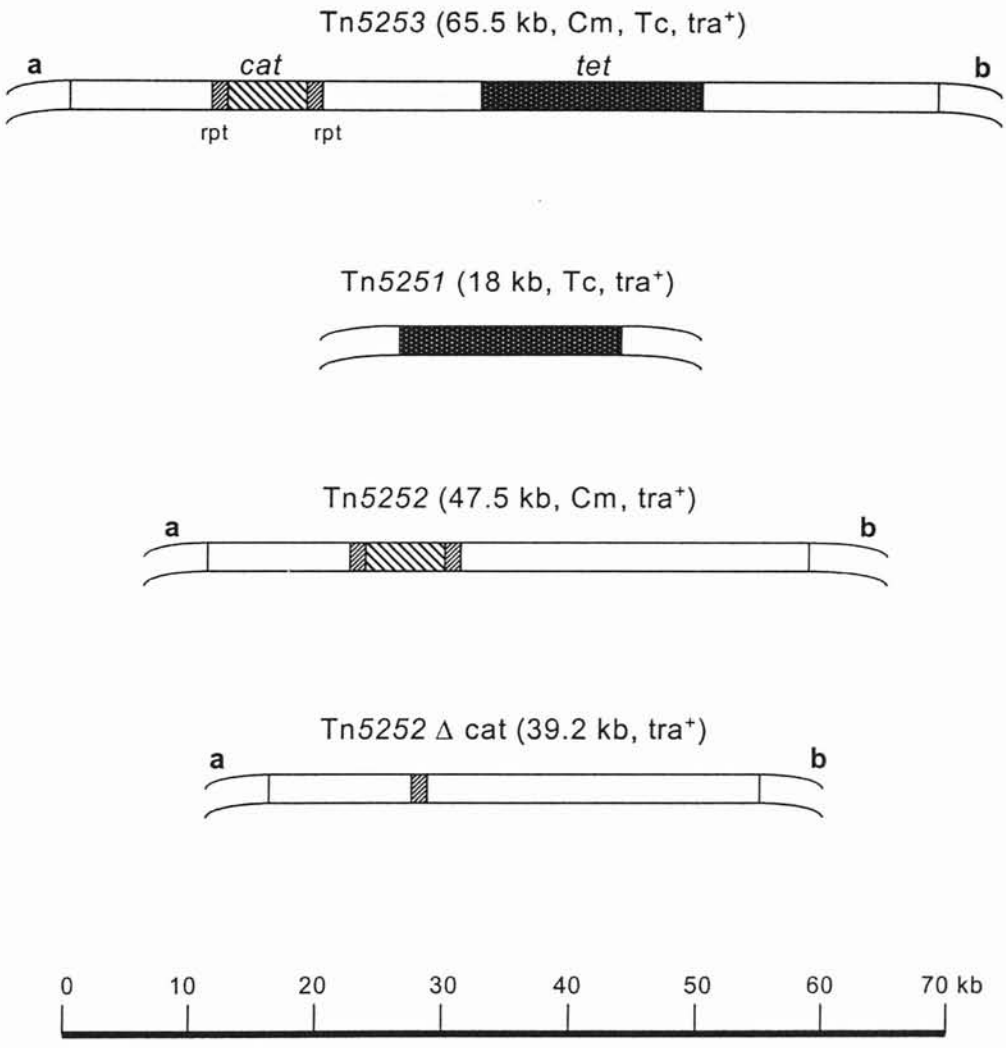

Figure 1. Composite conjugative transposon Tn*5253* and its derivative transposons Tn*5252* and Tn*5251*. The *cat* gene, flanked by 1.7 kb direct repeats, often spontaneously cures. However, Tn*5252*Δ*cat* is still transfer-proficient.

Among 13 open reading frames identified at the left terminal region, ORF1 and 2 were shown to carry a site-specific recombinase or integrase (83). ORF5, ORF 4 and ORF3 were identified as excisionase, DNA relaxase, and the regulator (Fig. 2), respectively. The regulator was shown to bind to upstream sequences of ORF1 and 2, ORF4 and to its own promoter (69). On the right region of Tn*5252* among 11 potential genes ORF6 was shown to be a DNA cytosine methyltransferase gene (64,65) (Fig. 3).

Tn*917* Insertion Mutagenesis

Tn*917* was first identified as a 5.3 kb transposon on *E. faecalis* DS16 plasmid pAD2 (89). The element is capable of enhanced transposition on exposure to low concentration of erythromycin (16). Although Tn*917* exhibits sequence similarity to *Staphylococcus aureus* transposon Tn*551*, it is grouped in the Tn*3* family. The members of Tn*3* family, including Tn*917*, generate a 5 bp duplication of target sequences upon insertion (73). Three major open reading frames of Tn*917* erm, *tnpA* and *tnpR* have been analyzed in detail. The *erm* gene is the erythromycin resistance determinant. The other two genes *tnpR* and *tnpA*, exhibit significant homology to Tn*3* family genes and, thus, they are presumed to encode for resolvase and transposase, respectively (89).

Transpositon of Tn*917* is an effective method of producing insertional mutants of *B. subtilis* and potentially other gram-positive and gram-negative bacteria. In order to understand the origins and properties of most of the Tn*917*

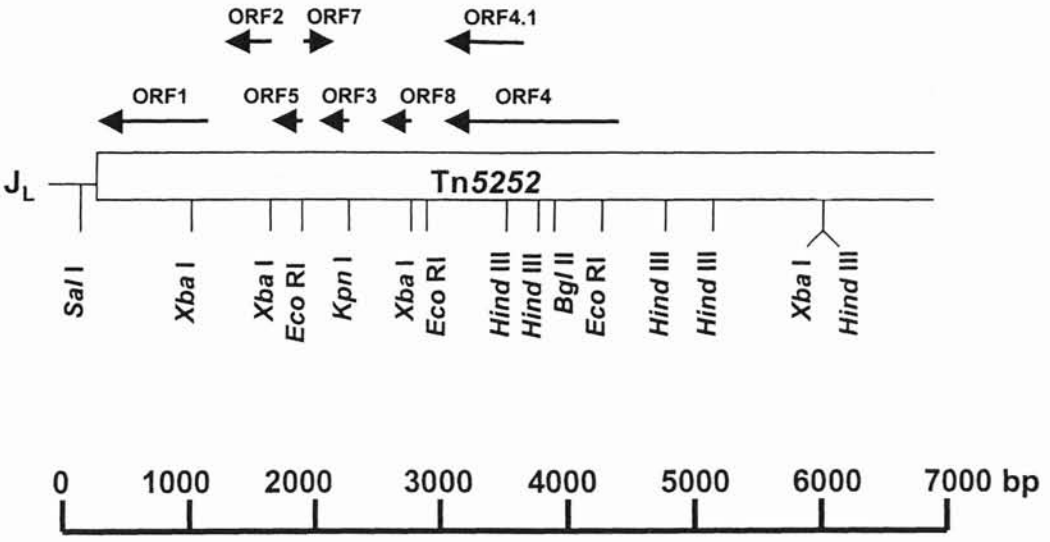

Figure 2. Restriction map of the left terminal region of Tn*5252* in *S. pneumoniae* SP1000. The direction and size of ORFs in this region are also shown.

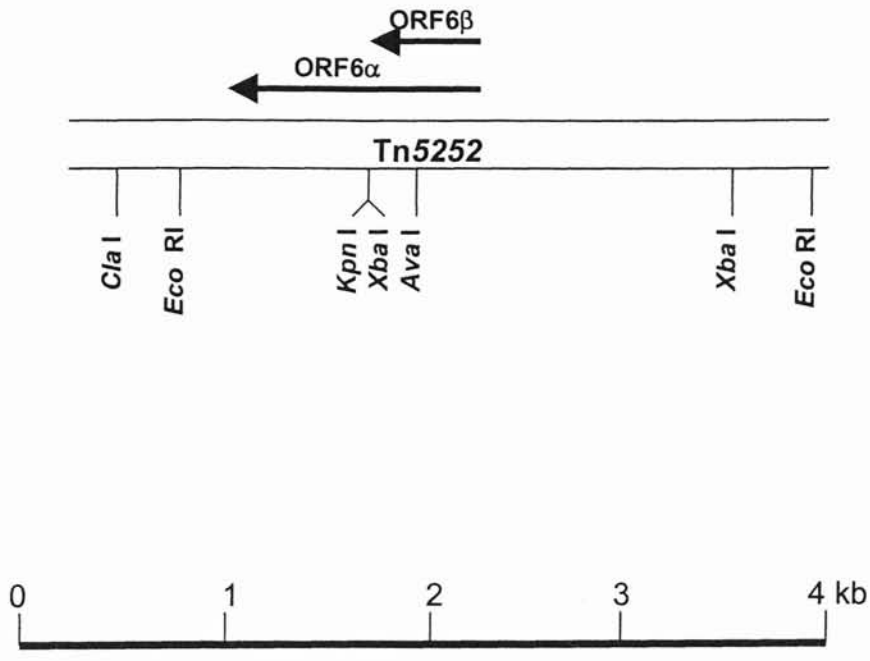

Figure 3. The restriction map of the right terminal region of Tn*5252* in *S. pneumoniae* SP1000. The direction and size of ORF6 is shown.

derivatives, it is important to mention that the interval between the *erm* and the nearest terminal repeat consists of nonessential DNA that may be modified without interfering with transposition (90). Although Tn*917* insertions are not perfectly random in their distribution over the *B. subtilis* genome, still insertions may be obtained in any chromosomal region and even within the coding sequence of any gene (90). Tn*917* insertion mutagenesis in *B.subtilis* was used to generate phenotypically cryptic mutants, auxotrophic mutations, and sporulation related mutations (90).

Tn*917* can be used as a mutagen in other gram-positive bacteria and even gram-negative bacteria. It has been shown that Tn*917* derivatives can actually function in *E. coli* (35). Evidently, this transposon has a potentially broad host range. On the other hand, several laboratories have exploited Tn*917* for physical and functional analyses of conjugative plasmids in streptococci, enterococci and staphylococci. The most successful application of this transposon was with the large functionally complex plasmids of *E. faecalis* to identify regions specifying plasmid transfer, pheromone response, conjugative transposition, drug resistance and hemolysin-bacteriocin activity (89). On the other hand, it was shown that Tn*917* insertions introduce mutations into the group B streptococci genome (25). Other successful applications of Tn*917* mutagenesis have been reported for *L.lactis* (33), *Streptococcus mutans* (21) and *Staphylococcus epidermidis* (27).

Insertion-Duplication Mutagenesis in *S. pneumoniae*

Transposons like Tn*917* are valuable mutagenic tools but they occasionally show some degree of target preference, which makes them poor candidates to be used to identify essential genes. An alternate method of mutagenesis is insertion-duplication mutagenesis (IDM), a widely used technique for analysis of specific genes (80).

S. pneumoniae is a representative of a diverse group of bacteria capable of natural genetic transformation. It can take up DNA and insert into its genome by homologous recombination upon induction of DNA uptake by an intracellular signaling peptide called "competence factor" (40). When competent pneumococcal cells are transformed using a linear homologous DNA donor, as a result of recombination process a linear chromosomal region will be replaced by a segment of the donor DNA. However, if the donor DNA is a chimeric circle comprising a homologous sequence to the recipient genome, the result can be the insertion of the entire circular DNA bounded by a duplication (Fig. 4 and 5). The heterologous region on the circular donor DNA is often a nonreplicative plasmid with a marker (40, 41). If the homologous targeting sequence of the circular donor DNA is totally internal to a gene then the inactivation of gene may take place (41). The marker on the heterologous region of the donor DNA is essential for the isolation of resulting mutants by selection.

Insertion-duplication mutagenesis is valuable as a mutagen due to the creation of highly random insertions as well as its use for specific targeting of

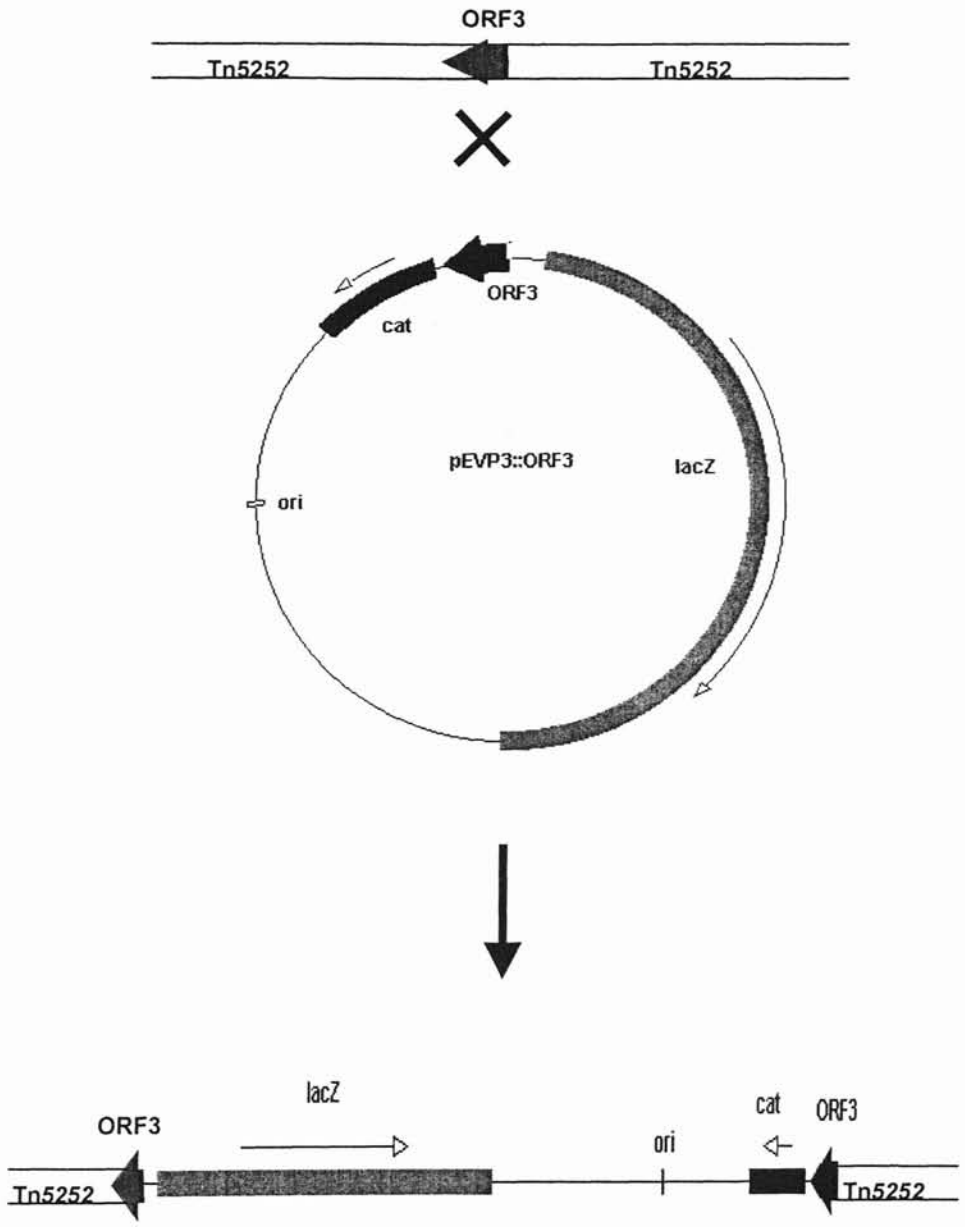

Figure 4. Insertion-duplication mutagensis. The open reading frame, i.e. ORF3 in Tn*5252*, is inserted into Tn5252, in the opposite direction as compared to *lacZ*. This orientation is designated as the reverse orientation.

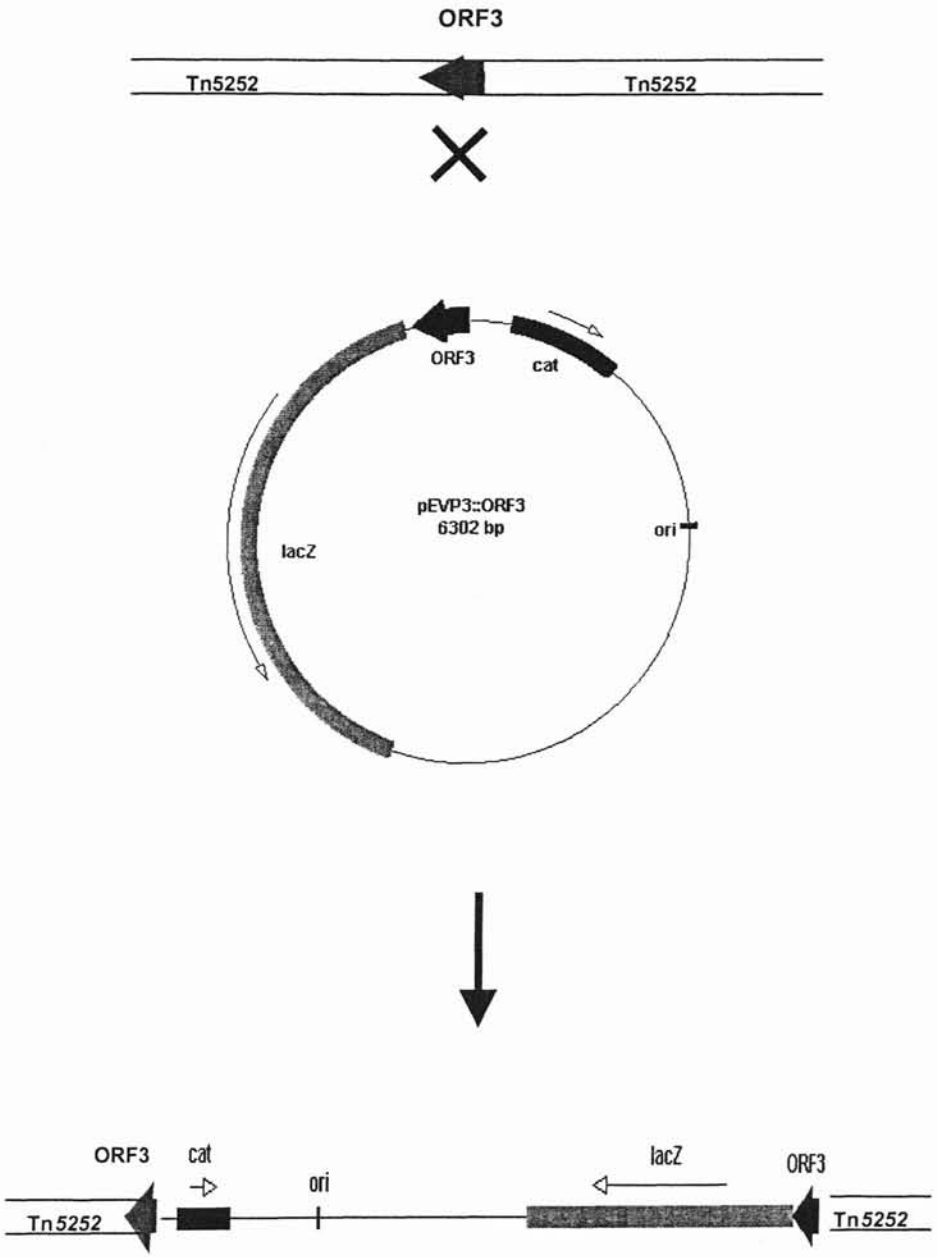

Figure 5. Insertion-duplication mutagenesis. The open reading frame, i.e. ORF3 in Tn*5252*, is inserted into Tn5252, in the same direction as compared to *lacZ*. This orientation is designated as the right orientation.

selected genes (40,52). If the chimeric circular DNA construct includes a *lacZ* reporter in addition to an antibiotic resistance marker, such molecules could be used to monitor the activity of the target gene by creating transcriptional or translational fusions (13).

Several research groups have mutated different *S. pneumoniae* strains by using insertion-duplication mutation strategy to study the function and regulation of pneumococcal chromosomal genes.

Recently, Holden et al. have identified several novel virulence loci in type 3 pneumococci (38) using insertion-duplication mutagenesis. Over a thousand mutant strains were analyzed for their ability to survive in mice models and among 186 mutant strains, 56 were selected for further genetic analysis based on their ability to excise the integrated plasmid spontaneously. The plasmids containing the genomic DNA inserts were cloned in *E. coli* and sequenced. After database search, 42 new virulence genes were identified but no pathogenicity islands were found.

Other research laboratories used insertion-duplication mutations to inactivate certain genes. These studies basically focused on the virulence factors and antibiotic resistance genes.

Pneumolysin gene, one of the proven virulence genes in *S. pneumoniae*, was mutated using IDM (3). It was shown that inactivation of pneumolysin gene in pathogenic pneumococcal strains (type 2 and type 3) reduced virulence of *S. pneumoniae* for mice challenged by the intranasal or intraperitoneal route (3).

Also, Vollmer and Tomasz reported the inactivation of pneumococcal teichoic acid phosphorylcholine esterase (*pce*) gene of *S. pneumoniae* by IDM (84). It was demonstrated that this type of mutation resulted in a unique change in colony morphology and a striking increase in virulence in mouse model.

Finally, the experiments by Filipe and Tomasz showed that the inactivation of *murMN* operon (encode cell wall muropeptides) in penicillin resistant *S. pneumoniae* strains resulted in correction of cell wall abnormality and complete loss of penicillin resistance. These results indicate that functioning of *murM* and *murN* genes is an integral component of penicillin-resistance mechanism in pneumococci (24).

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains and Plasmids

All *E. coli* strains used in this study are shown in Table 1. Recombination deficient strains of *E.coli*, JM109 and DH5α, were used for generation and amplification of recombinant plasmids. The recombination proficient *E.coli* strain C600 was used to amplify the vector plasmid pLS1.

Strains of *S.pneumoniae* used in this study were derivatives of the nonencapsulated wild type strain R×1 (75). Strain SP1000 is an R×1 derivative carrying Tn*5252*. CP1250 strain is a β-gal$^-$ R×1 derivative carrying a point mutation that confers β-gal$^-$ phenotype (2). SP1400 is a derivative of SP1000 carrying a chromosomal point mutation conferring β-gal$^-$ phenotype. SP1000 was transformed with CP1250 chromosomal DNA, transformants were selected on X-gal containing plates and among a few white colonies one was chosen and named as SP1400. SP1403 is a derivative of SP1400, lacking the *cat* gene after "curing". *E. faecalis* strains were used in electroporation experiments especially due to the absence of their β-galactosidase activity. JH2-2 is the wild type plasmid free

Table 1

Bacterial Strains

Strain	Genotype	Reference/Source
E.coli		
DH5α	ϕ80 *lacZΔM15 recA1 endA1 gyrA96 thi-1 hsDR17* (r_k^-, m_k^+) *supE44 relA1 deoR ΔlacYU169*	Bethesta Research Laboratories
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiA*, (lacproAB)(F' traD36 proAB+ *lacI*^{q} *lacZ*ΔM15)	(87)
C600	*sup*E44 *hsd*R *thi*-1 *thr*-1 *leu*B6 *lac*Y1 *ton*A21	
XLI-Blue MRA	Δ(mcrA)183, Δ(mcrCB-hsd SMR-mrr)173	
S.pneumoniae		
SP1000	*str*-1 *fus* Tn*5252* (*cat*)	(75)
SP1400	*str*-1 *fus* β-gal$^-$ Tn*5252* (*cat*)	This study
SP1403	*str*-1 *fus* β-gal$^-$ Tn*5252* Cm^s	This study
SP1501-1 to SP1501-6	*str*-1 *fus* β-gal$^+$ Tn*5252* pEVP3ΩORF3 (Cm^r)	This study
SP1502-1 to SP1502-5	*str*-1 *fus* β-gal$^-$ Tn*5252* pEVP3ΩORF4 (Cm^r)	This study
SP1503-1 to SP1503-6	*str*-1 *fus* β-gal$^+$ Tn*5252* pEVP3ΩORF6 (Cm^r)	This study
SP1504-1 to SP1504-4	*str*-1 *fus* β-gal$^+$ Tn*5252* pEVP3Ω ORF5 IntR (Cm^r)	This study
CP1250	hex$^-$ mal$^-$ str^r nov^s β-gal$^-$	(2)
E. faecalis		
JH2-2	Wild type, plasmid free	(86)
SF5002	*fus rif* Tn*5252*(*cat*)	(2)

E. faecalis strain. SF5002 is a derivative of *E. faecalis* UV202 carrying a Tn*5252* insert in the chromosome.

A list of plasmids used in this study is given in Table 2. One of the plasmids used in our experiments was pLS1, a 4.4 kb shuttle vector. pLS1 contains tetracycline resistance determinant and is used for molecular genetic studies with both gram-positive and gram-negative bacteria. Normal replication of pLS1 requires RecA function. Thus, *rec*$^+$ host strains are used to propagate this plasmid (66).

The vector plasmid, pAT29, is a shuttle vector used for cloning experiments in both *E. coli* and gram-positive bacteria. This plasmid confers resistance to spectinomycin and contains a multiple cloning site embedded in *lacZ*α gene (79).

A 9.2 kb plasmid, pVA838 is used as a shuttle vector to clone streptococcal fragments in *E. coli* (43,47). This plasmid contains resistance genes for erythromycin and chloramphenicol.

The pTV1 derivative plasmids, pTV32Ts and pTV53Ts are used to introduce Tn*917* insertion mutation in *B. subtilis* as well as other gram-negative and gram-positive bacteria (90). Both pTV32Ts and pTV53Ts carry a promoterless *E. coli lacZ* coding sequence that can generate transcriptional *lacZ* fusions. Additionally, pTV53Ts contains a promoterless *cat*-86 gene downstream of *lacZ* coding sequence with no transcriptional terminator between these genes. The plasmid pTV32Ts carries a *cat* gene as a selectable marker whereas

Table 2

Plasmids

Plasmid	Characteristics	Reference/Source
pLS1	4.4 kb, Tet^r shuttle vector for both Gram-positive and Gram-negative bacteria	(66)
pAT29	6.7 kb *lacZ* Spc^r, *E.coli*-Streptococcus shuttle vector	(79)
pVA838	9.2 kb, Em^r Cm^r, *E. coli-S. sanguis* shuttle vector	(43,47)
pTV53Ts	16.9 kb, Tn*917lacZ* Em^r Cm^r Tet^r	(90)
pTV32Ts	15.6 kb, Tn*917lacZ* Em^r Cm^r	(90)
pEVP3	6.3 kb, *lacZ cat*, *lacZ* reporter insertion vector	(13)
pEB2	*Kpn*I cut self-ligated plasmid from SP1501-1	This study
pEB3	*Kpn*I cut self-ligated plasmid from SP1501-2	This study
pEB4	*Kpn*I cut self-ligated plasmid from SP1503-2	This study
pEB5	*Eco*RI cut self-ligated plasmid from SP1501-1	This study
pEB6	pEVP3::ORF3, *Eco*RI cut 8 kb self-ligated plasmid from SP1501-1 with ORF3 inserted in the right orientation	This study
pEB7	pEVP3::ORF3, *Bam*HI cut 10 kb self-ligated plasmid from SP1501-1 with ORF3 inserted in the right orientation	This study
pEB8	*Bam*HI cut self-ligated plasmid from SP1501-1	This study
pEB9	*Bam*HI cut self-ligated plasmid from SP1501-1	This study
pEB10	pEVP3::ORF6, *Eco*RI cut 11 kb self-ligated plasmid from SP1503-2 with ORF6 inserted in the right orientation	This study
pEB11	*Eco*RI cut self-ligated plasmid from SP1503-2	This study

pTV53Ts codes for a tetracycline resistance marker outside the Tn*917* sequence.

The final vector plasmid used in this study, pEVP3, is a 6.3 kb insertion duplication vector plasmid for *S. pneumoniae* (13). The vector contains a promoterless *lacZ* gene as the reporter gene and a *cat* gene conferring chloramphenicol resistance. It also contains unique restriction sites between *lacZ* and *cat* gene useful for cloning purposes.

Growth Conditions and Media

E. coli strains were propagated at 37°C in Luria-Bertani (LB) broth with aeration and supplemented with the suitable antibiotics to maintain recombinant plasmids. Cultures were stored in 90% (v/v) LB broth and 10% (v/v) glycerol at -80°C.

S. pneumoniae and *E. faecalis* were grown, at 37°C without aeration, in CAT broth containing 1% (w/v) casein hydrolysate, 0.5% (w/v) tryptone, 0.1% (w/v) yeast extract, and 0.5% (w/v) NaCl. After sterilization, CAT broth was supplemented with 0.5% (w/v) glucose and 15 mM K_2HPO_4 for buffering. CTM was prepared the same as CAT broth except it was supplemented with 10 mM $CaCl_2$ and 0.2% bovine serum albumin. Since pneumococci tend to autolyse if grown to high densities, they were routinely grown to a maximum OD_{550nm} of 0.2 (ca. 2×10^8 CFU/ml). Cultures were stored in 90% (v/v) CAT broth and 10% (v/v) glycerol at -80°C.

Solid medium was prepared by adding 1.5-2.0% agar to the broth when needed. In order to provide anaerobic conditions *S. pneumoniae* strains were grown between CAT agar layers supplemented with 2% (v/v) bovine or sheep blood. *E. coli* and *E. faecalis* strains were plated on LB agar and CAT agar surfaces, respectively. Antibiotic concentrations for selection of bacterial strains are given in Table 3.

Chemicals, Restriction Endonucleases and Media

Bacteriological agar and media were obtained from Difco or Fisher. Antibiotics, DNase I, RNase I, egg white lysozyme, bovine serum albumin were purchased from Sigma Chemical Co. Restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase I, Deep Vent DNA polymerase and DNA molecular weight standards were purchased from Promega Corp., Bethesda Research Laboratories (BRL) or New England Biolabs (NEB) and used as described by the manufacturer. IPTG (isopropyl-β-D thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D galactoside) were obtained from BRL whereas ONPG (*o*-nitrophenyl--β-D galactoside) was purchased from Sigma Chemical Co. Agarose from Fisher and ultrapure agarose from Bio-Rad Laboratories were used for agarose gel electrophoresis and electroelution. All other chemicals, compounds and reagents were purchased from either Sigma or Fisher.

Table 3

Antibiotic Concentrations

Phenotype	Antibiotic	Concentration (μg/ml) stab plate/broth	overlay
E. coli			
Cm	chloramphenicol	10	
Tet	tetracycline	10	
Ery	erythromycin	200	
Aph	kanamycin	50	
Amp	ampicillin	50	
Spc (pAT29)	spectinomycin	150	
S. pneumoniae			
Cm	chloramphenicol	5	15
Ery	erythromycin	0.5	0.5
Fus	fusidic acid	10	50
Nov	novobiocin	10	10
Rif	rifampicin	10	10
Str	streptomycin	200	200
Tet (Tn*5253*)	tetracycline	2	5
Spc (pAT29)	spectinomycin	200	350
Erm	erythromycin	3	5
E. faecalis			
Cm	chloramphenicol	25	
Fus	fusidic acid	25	
Rif	rifampicin	25	
Str	streptomycin	200	
Tet	tetracycline	4	
Spc (pAT29)	spectinomycin	250	

Transformation

Transformation of *E.coli*

Recombinant plasmids containing Tn*917* fragment and pEVP3 derivative plasmids were used to transform competent cells according to the methods described by Hannahan (29). Transformants were isolated by plating several dilutions of the transformation mixture on selective LB agar plates. IPTG (isopropyl-β-D thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D galactoside) were added to selective plates for phenotypic differentiation.

Transformation of *E. faecalis*

Electrocompetent *E. faecalis* cells were prepared and were transformed by electroporation using a modified version of Fiedler's and Wirth's protocol (23). Cells were grown overnight in CAT broth supplemented with potassium phosphate (dibasic) and glucose. After harvesting the cells by centrifugation, they were washed three times in chilled 10 % glycerol in distilled water with 1/1, 1/2 and 1/10 original volume of the growth medium. After the final wash, cells were resuspended in chilled 10 % glycerol in 1/1000 original volume of growth medium. Electrocompetent cells were frozen and stored at -80°C for upto two months. 40 μl of cells and about 200 ng of plasmid DNA were mixed, transferred to a chilled 0.2-cm electroporation cuvette (Invitrogen) and incubated at 0°C for 5

minutes. Cells were pulsed immediately at 1250 V and 360 Ω using an Electro Cell Manipulator 600 (BTX Electroporation System). After electroporation, cells were transferred to pre-chilled 1 ml of CAT broth and incubated at 0°C for 10 minutes. Then they were incubated at 37°C for 90 to 120 minutes to allow time for phenotypic expression of antibiotic resistance genes. After incubation cells were spread on CAT plates supplemented with appropriate antibiotics.

Transformation of *S. pneumoniae*

Competent cells of pneumococcus were prepared according to the method described by Guild and Shoemaker (28). When needed competent cells were thawed on ice, mixed with donor DNA (1 μg/ml for chromosomal DNA and 10 μg/ml for plasmid DNA) and incubated at 37°C for 30 minutes. Then pancreatic DNase I solution in CAT broth was added at a final concentration of 10 μg/ml and the cultures were incubated 5 more minutes at 37°C. Transformant cells were transferred on ice and appropriate dilutions were plated on CAT agar using the overlay method. The plates were incubated at 37°C for 18 to 24 hours.

S. pneumoniae Transformation Using Competence Factor

Lyophilized preparation of competence factor, CSP-1, was dissolved and diluted in 50 mM potassium acetate buffer (pH: 4.6) at a final concentration of 100 μg/ml. 1 ml of CTM broth containing potassium phosphate, glucose, $CaCl_2$,

BSA and CSP-1 (at a final concentration of 1μg/ml) was mixed with 20 μl of donor DNA and 50 μl of cells at an OD_{550} of 0.2 (53,11). The cells were incubated at 37°C for 90 minutes. DNase I was added to the cultures at a final concentration of 50 μg/ml and they were incubated at 37°C for 30 minutes. Cells were plated as described for natural transformation.

DNA Isolation

Plasmid DNA Isolation from *E. coli*

Alkaline-SDS lysis and rapid plasmid DNA isolation from *E.coli* were performed according to Birnboim et al. and Colman et al. (4). Plasmid DNA was further purified with equal volumes of phenol-chloroform, and chloroform-isoamylalcohol, before ethanol precipitation, and resuspended in 50 μl of TE buffer or sterile distilled water.

An alternate method used to isolate small scale plasmid DNA from *E. coli* was Wizard Column DNA Preparation Protocol (Promega).

Large scale plasmid DNA purification was essentially performed the same as alkaline-lysis plasmid DNA purification protocol except for the scale-up preparation. The lysate was mixed with cesium chloride and ethidium bromide and treated as described by Sambrook et al. (63).

Chromosomal DNA Isolation from *S. pneumoniae*

Pneumococcal strains were grown in 200 ml CAT broth supplemented with potassium phosphate (33%) and glucose (1%). After growing the cells to an OD_{550nm} of 0.3-0.4, cultures were mixed with 10 ml 10 mM EDTA and kept on ice for 10 minutes. Cells were washed twice with 50 mM Tris-HCl, 20 mM EDTA, pH 7.5 and pelleted by centrifugation at 5,000×g in a Sorvall RC-5B centrifuge (DuPont Instruments). Cell pellets were resuspended in 5 ml of the same buffer followed by addition of 1 ml of a solution containing 0.6% triton X-100, 0.06% sarkosyl, 0.6% sodium deoxycholate (DOC) and RNase (300 μg/ml). The cell suspension was incubated at 37°C until lysis was visible. Lysis was completed by adding 1 ml of 1% SDS and Proteinase K (350 μg/ml) and by overnight incubation at 65°C. DNA was further purified by phenol-chloroform extraction and following ethanol precipitation DNA was dried and resuspended in 400 μl of TE buffer.

Molecular Cloning Techniques

DNA digestions and ligations were done as described by Sambrook et al (59). For insertion duplication mutagenesis in *S. pneumoniae*, the insert:vector ratio was 5:1 in a 25 μl total volume of ligation mixture. Ligation reaction was performed for 1-2 days at 16°C and continued 2 more days at 4°C. Ligated DNA was used as donor DNA for transformation of competent *S. pneumoniae* cells.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed as outlined by Sambrook et al (63). Plasmid and chromosomal DNA samples were prepared with a tracking dye solution (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 20% glycerol, 0.1 M EDTA, pH 8.0) and loaded onto a 0.8% or 1.2% agarose gel (Fisher) with a molecular size marker (*Hind* III fragments of lambda phage or 1kb ladder). Electrophoresis was routinely carried out in TBE buffer (89 mM Tris Base, 89 mM boric acid and 2.5 mM EDTA, pH 8.0) at room temperature. After electrophoresis gels were stained with 1.0 μg/ml ethidium bromide for 10 minutes and destained in deionized water for 20 minutes. DNA bands were visualized on the gels using an UV transilluminator and photographed using Alpha Imager 2000 (Alpha Innotech Corporation).

Specific DNA fragments were purified using 0.8% agarose gels made with UltraPure agarose (Bio-Rad). After staining with ethidium bromide DNA fragment was electroeluted into a dialysis bag and purified essentially as described by Sambrook et al (63).

Southern Hybridization

The Southern hybridization was performed following the methods described by Southern (71) and according to manufacturer's recommendations. After electrophoresis on 0.8 % agarose gel, the DNA was denatured by soaking the gel in 200 ml of 0.5 N NaOH for 30 min. The DNA was transferred to a nylon

membrane (GeneScreen Plus, NEN) using a vacuum blotter (Model 785 Vacuum Blotter, Bio-Rad) for 90 min at 5 Hg/inch. The DNA-bound membrane was prehybridized at 65°C for more than 6 h in a hybridization oven (Techne Hybridiser HB-2D). Probes were labeled with (α-^{32}P) dATP (NEN) by using the nick translation protocol described by Sambrook et al (14). The heat denatured probe was added to 1 to 4×10^6 cpm for each hybridization. Hybridization was carried out at 65°C for 18 h. The membrane was washed, air dried and exposed to X-ray film (Kodak X-OMAT-AR) at - 80°C for varying time periods.

β-Galactosidase Assay

Cultures of insertion-duplication mutants were grown in 10 ml CAT-PG broth to an OD_{550} of 0.2. Cells were collected by centrifugation 5000 rpm for 10 min and the pellet was resuspended in 0.5 ml CAT-PG broth. Cells were lysed by adding 100µl of 1% Triton-X-100 and incubated at 0°C and 37°C for 10 min. the lysate was kept at 0°C until needed for the assay. The enzyme reaction was started at room temperature by adding 0.5 ml Z buffer and 0.2 ml ONPG (*o*-nitrophenyl--β-D galactoside) at a final concentration of 4 mg/ml. After overnight incubation in dark, reaction was stopped by adding 0.5 ml 1M Na_2CO_3 (prepared fresh). OD was measured at 420 nm and 550 nm. Enzyme activity was expressed in Miller units with respect to the OD of culture at 550 nm (48).

CHAPTER IV

CONSTRUCTION OF A β-GAL REPORTER SYSTEM TO IDENTIFY CONSTITUTIVELY VERSUS INDUCIBLY EXPRESSED GENES IN Tn*5252* USING Tn*917* MUTAGENESIS

RESULTS

Tn*917* mutagenesis has been proven to be a powerful tool for genetic analysis of several medically important bacteria including *S. pneumoniae* (1, 2, 3). In this study we were interested in integrase (ORF1 and 2), excisionase (ORF5), DNA relaxase (ORF4), regulator (ORF3), and DNA-cytosine methyltransferase (ORF6) genes in Tn*5252*. To analyze the nature of gene expression, different vector plasmids were employed to introduce a reporter *lacZ* gene downstream the genes of interest in Tn*5252* via random insertion of Tn*917*Ω*lacZ*. In order to have a promoterless *lacZ* gene insert we attempted to clone a 12.6 kb Tn*917*Ω*lacZ* fragment of pTV53Ts (Fig. 6) into the *Eco*RI site of the vector plasmids pLSI (4), pVA838 (5) and pAT29 (6). The 12.6 kb Tn*917*Ω*lacZ* fragment contains a promoterless *lacZ* gene, *cat* and *erm* antibiotic resistance markers.

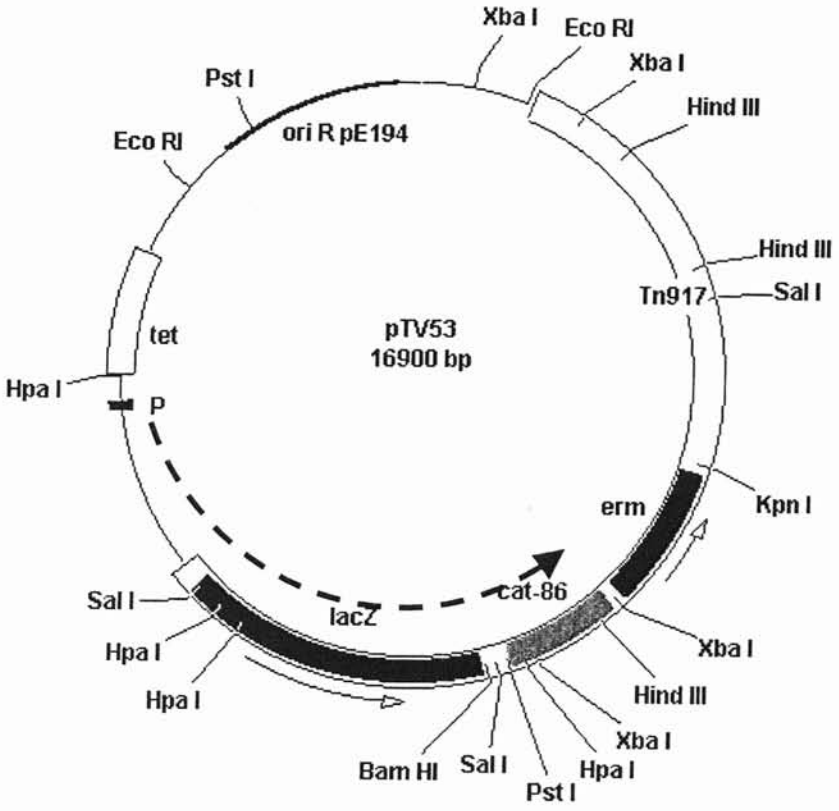

Figure 6. Map of pTV53Ts (90).

Tn*917* Mutagenesis Using pLSI

Initially, the broad-host range plasmid, pLS1, was chosen as the vector plasmid because of its capability to replicate and confer tetracycline resistance in both gram-positive and gram-negative bacteria (Fig. 7). The plasmid has high copy number in *S. pneumoniae* hosts and replication of streptococcal plasmids in *E. coli* requires *recA* function. In order to construct a pLS1::Tn*917ΩlacZ* recombinant plasmid, the 12.6 kb Tn*917ΩlacZ* fragment and *Eco*RI cut pLS1 were ligated. The ligation mixture was used to transform *E. coli recA*$^+$ host strain, C600. After transformation we selected for Tc^r and Em^r transformants (tet^r gene is in pLS1 and erm^r gene in Tn*917ΩlacZ*). Despite of our efforts, no Tc^r and Em^r transformants were isolated. On the other hand, when *E. coli* C600 was transformed by pLS1 as the donor DNA, the number of isolated Tc^r transformants was 1800 (Table 4).

To clone the Tn*917ΩlacZ* fragment into pLS1 we also transformed *S. pneumoniae* host strains SP1000 and CP1250 using pLS1 and pLS1:: Tn*917ΩlacZ* ligation mixture. When pLS1 was transferred to SP1000, the number of isolated Tc^r transformants was 63000. Similarly we obtained 4000 Tc^r transformants when CP1250 was transformed using pLS1 (Table 4). However, there were no Tc^r and Em^r transformants when SP1000 and CP1250 were transformed with pLS1::Tn*917ΩlacZ* ligation mixture. Transformation experiments performed by using *E. coli* C600 and, *S. pneumoniae* SP1000 and

Table 4

The "clonability" of Tn*917ΩlacZ* on Various *E. coli* Vector Plasmids

Host Strain	Donor DNA	Number of Transformants
E. coli JM109	pVA838	300 (Em^r)
	pVA838::Tn*917ΩlacZ*	3 (Em^r)
	pAT29	2300 (Spc^r)
	pAT29::Tn*917ΩlacZ*	2000 (Spc^r)
E. coli C600	pLSI	1800 (Tc^r)
	pLSI::Tn*917ΩlacZ*	-
E. coli DH5α	pAT29	3200 (Spc^r)
	pAT29::Tn*917ΩlacZ*	2580 (Spc^r)
S. pneumoniae SP1000	pLSI	63000 (Tc^r)
	pLSI::Tn*917ΩlacZ*	-
S. pneumoniae CP1250	pLSI	4000 (Tc^r)
	pLSI::Tn*917ΩlacZ*	-
E. faecalis JH2-2	pAT29	750 (Spc^r)
	pAT29::Tn*917ΩlacZ*	170 (Spc^r)

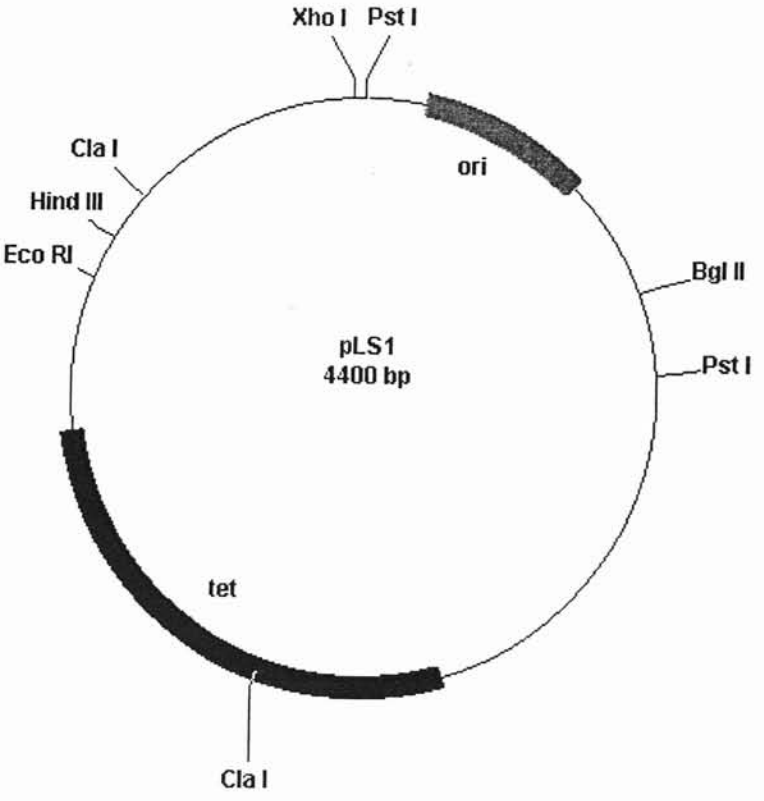

Figure 7. Map of pLS1 (66).

CP1250 as hosts showed that the recipient cells were competent considering the high number of Tc^r transformants when pLS1 was used as donor DNA. However, it is not possible to clone the 12.6 kb Tn*917ΩlacZ fragment* into pLS1, possibly due to the potential toxicity of Tn*917* in certain hosts.

In addition, pLS1 and pLS1::Tn*917ΩlacZ* ligation mixture were introduced into *E. faecalis* host strain JH2-2 by electroporation. After incubation a few white Tc^r transformants were isolated on X-gal containing plates, but there were no Em^r transformants. Plasmid DNA isolated from Tc^r JH2-2 transformants was analyzed by restriction enzyme digestion and the observed DNA band patterns confirmed the presence of pLSI.

Our results show that cloning of the 12.6 kb Tn*917ΩlacZ* fragment into pLS1 is not possible. As a result we employed a different strategy in which we used a new vector plasmid that could be useful to clone Tn*917ΩlacZ* fragment.

Tn*917* Mutagenesis Using pVA838

The plasmid pVA838 is a shuttle vector that has been shown to be useful in cloning streptococcal plasmid fragments in *E. coli* (Fig. 8). It expresses Em^r in both *E. coli* and *Streptococcus* spp. Its Cm^r marker is only expressed in *E. coli* and may be inactivated by DNA insertion at its internal *Eco*RI site.

In order to clone the 12.6 kb Tn*917ΩlacZ* fragment, pVA838 was cut at *Eco*RI site which is internal to its *cat* gene. Tn*917ΩlacZ* fragment was ligated to *Eco*RI cut pVA838. *E. coli* host strain JM109 was transformed with pVA838 and

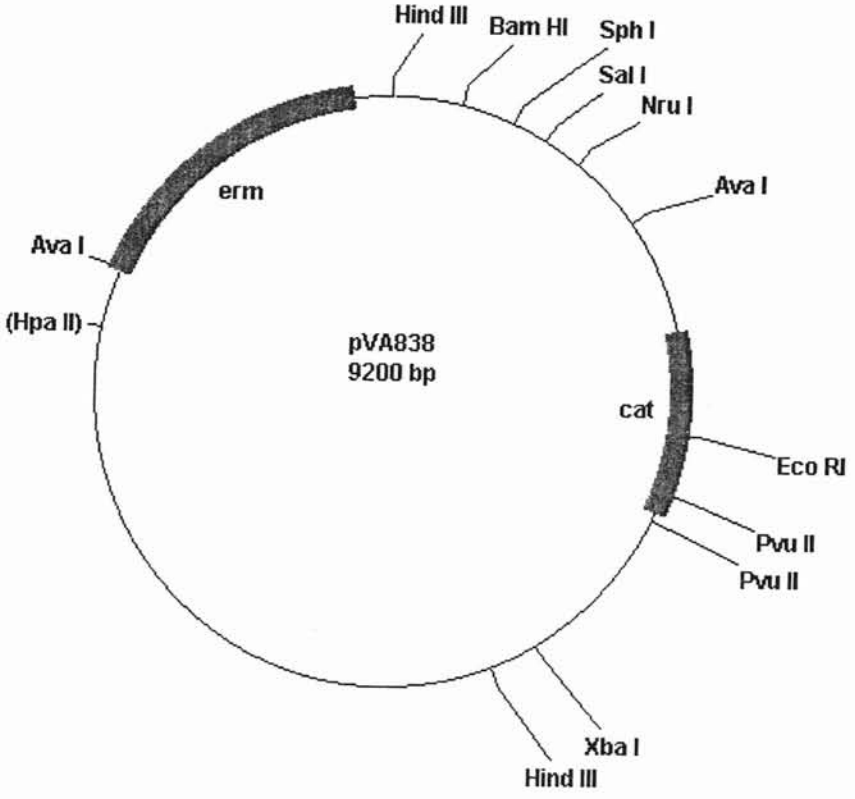

Figure 8. Map of pVA838 (43,47).

pVA838::Tn*917ΩlacZ* ligation mixture. The number of isolated Em^r transformants was 300 when pVA838 was used as donor DNA (Table 1). However, when pVA838::Tn*917ΩlacZ* ligation mixture was transferred to JM109 we isolated only three Em^r transformants. These Em^r transformants were replica-plated on erythromycin and chloramphenicol containing plates to find out if Tn*917ΩlacZ* fragment is cloned at *Eco*RI site on pVA838. Growth was observed on both erythromycin and chloramphenicol containing plates implying that *cat* gene was still intact. Thus, Tn*917ΩlacZ* was not inserted into pVA838. The results described above implied that Tn*917ΩlacZ* fragment cannot be cloned into pVA838.

Tn*917* Mutagenesis Using pAT29

The shuttle vector pAT29 was employed as the vector plasmid on our final attempt to mutagenize Tn*5252* using Tn*917* mutagenesis (Fig. 9). The plasmid pAT29 is conferring spectinomycin resistance and used for molecular cloning in *E. coli* and in gram-positive bacteria. *E. coli* host strains JM109 and DH5α were transformed with pAT29 and pAT29::Tn*917ΩlacZ* ligation mixture. After incubation we isolated 2300 Spc^r transformants when pAT29 was transferred to *E. coli* JM109 (Table 1). Similarly, when the ligation mixture was used as donor DNA the number of *E. coli* JM109 Spc^r transformants was 2000. However, DNA analysis showed that all the transformants had only pAT29 plasmid DNA.

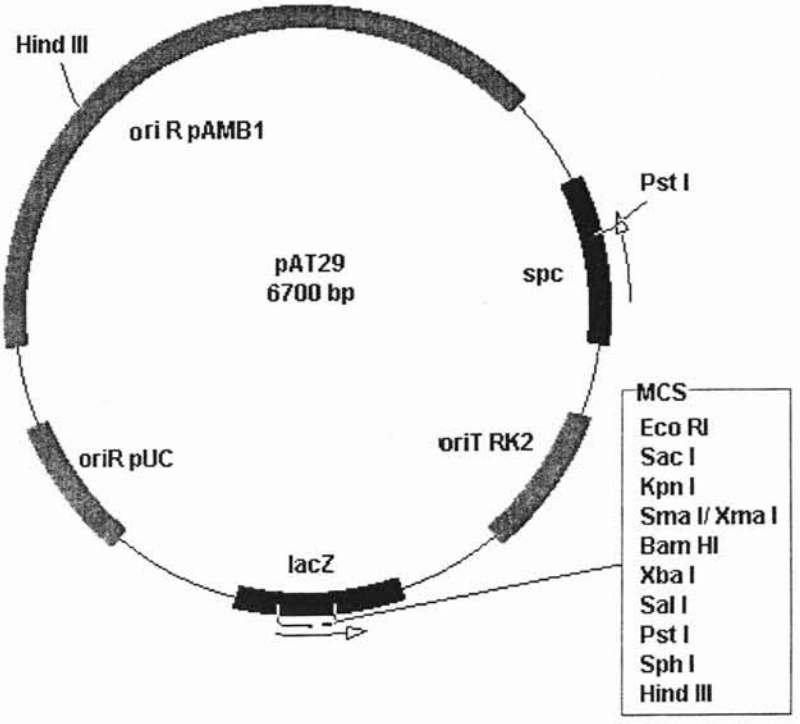

Figure 9. Map of pAT29.

On the other hand, DH5α transformation yielded 2580 Spc^r transformants when the ligation mixture was used as donor DNA whereas the number of Spc^r transformants for pAT29 donor DNA was 3200.To find out if the 12.6 kb Tn*917ΩlacZ* fragment was cloned into pAT29 at its *Eco*RI site, Spc^r transformants were replica-plated on tetracycline and erythromycin containing plates. Tc^r and Em^r are selective markers on the Tn*917ΩlacZ* fragment. None of the transformants could grow under tetracycline and erythromycin selection indicating that the 12.6 kb fragment was not cloned into pAT29. After restriction enzyme digestion analysis of plasmid DNA isolated from blue Spc^r transformants displayed pAT29 DNA fragment band pattern. Although the transformants were blue it wasn't due to the *lacZ* gene on the 12.6 kb fragment. Since replica plate results showed that DNA fragment was not cloned at multiple cloning site on pAT29. We can conclude that pAT29 *lacZ*α gene was intact. Therefore, a possible complementation may be taking place between *lacZ*α gene of pAT29 and *lacZ* gene on DH5α. As a result, the blue color of transformants is possibly due to the expression of *lacZ* gene on pAT29.

E. faecalis JH2-2 served as the recipient strain for the following transformation experiments. We isolated 750 Spc^r JH2-2 transformants when pAT29 was transferred by transformation. Whereas the number of *E. faecalis* JH2-2 Spc^r transformants for the ligation mixture was 170 (Table1). DNA was isolated from *E. faecalis* JH2-2 Spc^r transformants possibly containing the ligation mixture. Agarose gel electrophoresis analysis displayed smeared DNA bands implying that the DNA is possibly a mixture of plasmid and chromosomal

DNA. We observed high molecular weight DNA bands that are possibly as big as chromosomal DNA. To find out if the isolated DNA is a mixture of chromosomal DNA and plasmid DNA, *E. coli* host strain JM109 was transformed using the DNA isolated from *E. faecalis* JH2-2 Spc^r transformants. We isolated blue *E. coli* JM109 Spc^r transformants. After replica-plating and DNA analysis, it was shown that the transformants contained only pAT29 plasmid DNA. The reason for isolating blue colonies may be the complementation between the pAT29 *lacZ* gene and *lacZ*α fragment on JM109 chromosomal DNA.

It is concluded that pAT29 is not a suitable vector to clone Tn*917*Ω*lacZ* fragment due to the presence of *lacZ*α on this plasmid. Although beta-galactosidase$^-$ host strains were employed for transformation, we observed beta-galactosidase expression due to the *lacZ*α gene on pAT29. Replica plating and DNA analysis showed that beta-galacatosidase gene expression is not due to the *lacZ* gene on Tn*917*Ω*lacZ* fragment.

All the results presented in this section demonstrate that Tn*917*Ω*lacZ* fragment of pTV53Ts cannot be cloned into vector plasmids pLS1, pVA838 or pAT29, eventhough suitable *E. coli*, *S. pneumoniae* and *E. faecalis* host strains were employed in transformation experiments.

CHAPTER V

CONSTRUCTION OF A β-GAL REPORTER SYSTEM TO IDENTIFY CONSTITUTIVELY VERSUS INDUCIBLY EXPRESSED GENES IN Tn*5252* USING INSERTION-DUPLICATION MUTAGENESIS

RESULTS

Insertion-duplication mutagenesis (IDM) is a widely used mutagenesis technique for analysis of specific genes (50,13). It is a valuable tool for mutagenesis due to its use for specific targeting of selected genes. *S. pneumoniae* genome has been mutagenized using IDM (40,41). A donor DNA which is a chimeric circle comprising a homologous sequence (ORF3) to Tn*5252* is introduced into *S. pneumoniae* (Fig. 4 and 5). The heterologous region on the circular donor DNA is a nonreplicative plasmid (pEVP3) with a Cm^r marker. The result is the insertion of the entire circular DNA bounded by an ORF3 duplication. The orientation of insertion of the selected gene is important for evaluation of its expression by employing *lacZ* as a reporter gene.

Insertion-Duplication Mutagenesis Using pSJ126

Primarily we used a 3.9 kb plasmid, pSJ126, conferring kanamycin and

ampicillin resistance in *E. coli* (60). The plasmid has two identical mirror-image multiple cloning sites flanking the kanamycin resistance determinant which makes it useful for cloning purposes (Fig. 10). In order to create *lacZ* transcriptional fusions with specific genes on Tn*5252* and to use *lacZ* gene as a reporter we sought to replace the kanamycin gene with the *lacZ* fragment of pTV32Ts plasmid in pSJ126 (Fig. 11). The plasmid pTV32Ts was double-digested with restriction enzymes, *Sma*I and *Hind*III. A 5 kb *lacZ-erm* fragment was isolated by electroelution and, after klenow treatment, it was ligated to a 3 kb pSJ126 *Sph*I fragment purified by electroelution. *E. coli* host strain JM109 was transformed with pSJ126[*Sph*I]::*lacZ-erm* ligation mixture. A few transformants that could grow under ampicillin selection were isolated. However, no pSJ126[*Sph*I]::*lacZ-erm* plasmid DNA could be extracted. On the contrary, we were able to isolate self-ligated pSJ126 plasmid from *E. coli* JM109 Amp^r transformants. As a result of these transformation experiments we concluded that the *lacZ-erm* fragment from pTV32Ts can not be cloned into pSJ126.

Insertion-Duplication Mutagenesis Using *lacZ-erm* Fragment of pTV32Ts

A different strategy was used to insert the 5 kb *lacZ-erm* fragment downstream of different Tn*5252* genes. Klenow-treated *lacZ-erm* fragment was ligated to the following PCR products: ORF1, 2 and 5, ORF3, ORF4 and ORF6β. β-gal^- *S. pneumoniae* strain SP1400 was transformed with each of the ligated

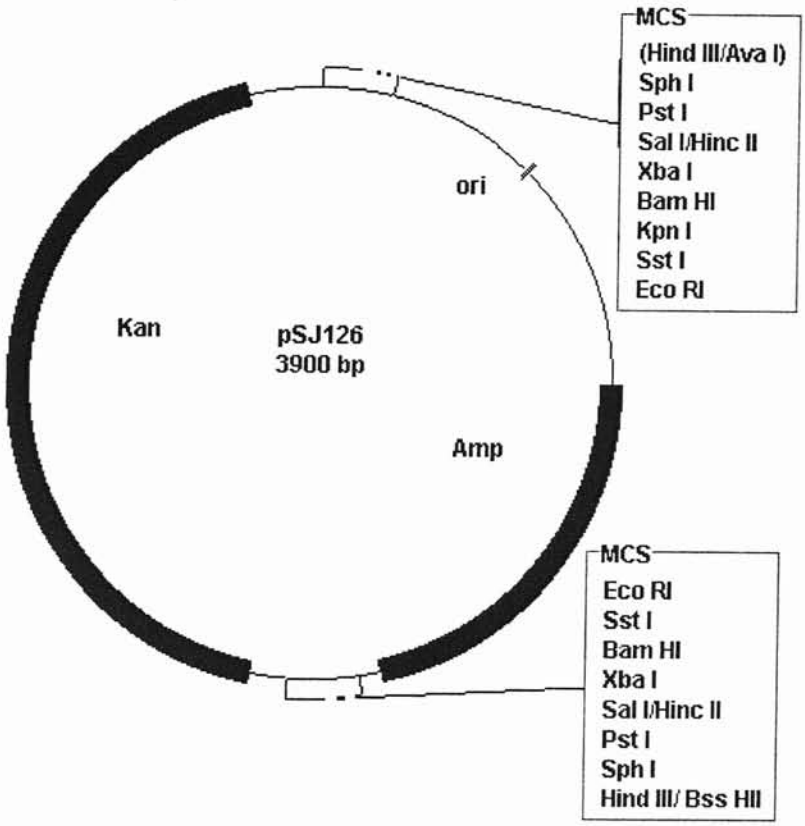

Figure 10. Map of pSJ126 (61).

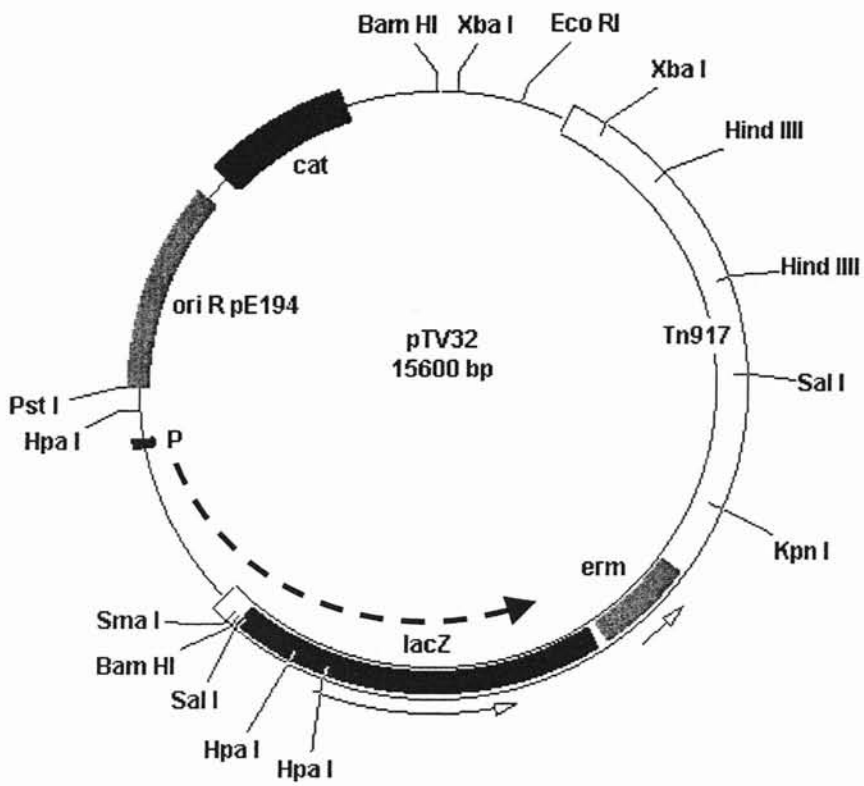

Figure 11. Map of pTV32Ts (90).

DNA. *S. pneumoniae* DP1617 DNA was used as a control for transformation. After transformation we isolated 2200 *S. pneumoniae* SP1400 Nov^r transformants implying that the recipient cells were competent. The amplified ORFs ligated to *lacZ-erm* were used as donor DNAs and transformants were selected on erythromycin plates. We isolated one Em^r transformant when *lacZ-erm*::ORF5 ligation mixture was used as donor DNA. We named this putative mutant strain as SP1401. Similarly, when *lacZ-erm*::ORF1, 2 and 5 was used as the donor DNA we could isolate only one Em^r transformant which was named as SP1402.

Putative mutant strains SP1401 and SP1402 were analyzed by Southern hybridization and β-galactosidase enzyme assay. On the autoradiogram DNA band patterns of both SP1401 and SP1402 were similar to the SP1403 control DNA (Fig. 17). Furthermore the β-galactosidase activity of both strains were significantly low (Table 6). Finally, *S. pneumoniae* R×1 and SP1403 strains were transformed using SP1402 DNA. We isolated no Em^r R×1 transformants. Similarly, no Em^r SP1403 transformants could be isolated. The number of SP1403 transformants should be several fold more than the number of R×1 transformants if the inserted DNA is in Tn*5252* region. These results indicated that there was no DNA inserted into Tn*5252*.

The results explained above show that pTV32Ts *lacZ-erm* fragment cannot be used to mutagenize specific Tn*5252* genes by employing insertion-duplication mutagenesis. Hence, experiments using lacZ-erm of pTV32Ts were aborted and we resorted to using another insertion vector plasmid, pEVP3.

Insertion Duplication Mutagenesis Using Insertion Vector Plasmid pEVP3

The new vector chosen for our studies was pEVP3, a 6.3 kb plasmid containing a promoterless *lacZ* gene as the reporter gene (Fig. 12). The plasmid confers chloramphenicol resistance expressed in both *E. coli* and *S. pneumoniae*. However, the plasmid is capable of replication only in *E. coli*.

To use the *cat* gene on pEVP3 as a selective marker for transformation experiments, SP1400 strain (that is Cm^r) was manipulated. The *cat* gene located on Tn*5252* was allowed to be spontaneously cured. A new β-gal$^-$ Cm^S *S. pneumoniae* strain, SP1403, was derived from SP1400. In our subsequent studies SP1403 was used as the host *S. pneumoniae* strain.

In order to clone selected Tn*5252* genes into pEVP3, the plasmid was cut with *Sma*I and ligated to promoterless ORF3, ORF4, ORF6 and ORF1, 2 and 5 PCR products. The recipient strain SP1403 was transformed with pEVP3 [*Sma*I]::ORF ligation mixture. The transformants were screened for chloramphenicol resistance. Six Cm^r transformants for pEVP3[*Sma*I]::ORF3 ligation mixture were isolated. These putative mutant strains were named as SP1501-1, SP1501-2, SP1501-3, SP1501-4, SP1501-5 and SP1501-6. Five mutant strains were isolated when pEVP3 [*Sma*I]::ORF4 ligation mixture was used to transform SP1403. These strains were named as SP1502-1, SP1502-2, SP1502-3, SP1502-4 and SP1502-5. Also, we isolated six strains when pEVP3[*Sma*I]::ORF6 was the ligation mixture used as donor DNA and these

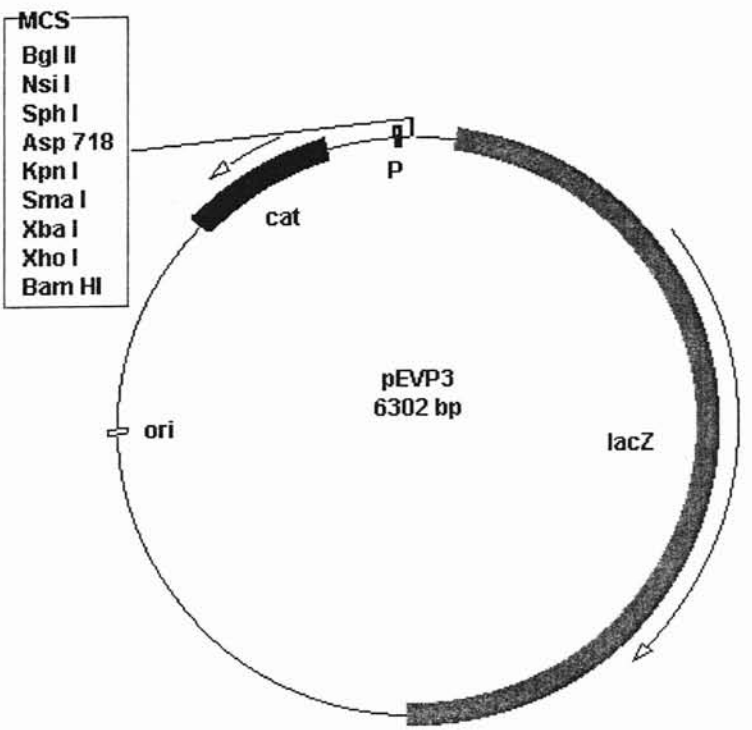

Figure 12. Map of pEVP3 (13).

strains were named as SP1503-1, SP1503-2, SP1503-3, SP1503-4, SP1503-5 and SP1503-6. Finally, four Cm^r transformants were isolated when pEVP3[*Sma*I]::ORF1, 2 and 5 ligation mixture was used as the donor DNA. These strains were designated SP1504-1, SP1504-2, SP1504-3 and SP1504-4. After isolating several transformants we wanted to find out if pEVP3[*Sma*I]::ORF chimeric molecule was inserted into Tn*5252* on SP1403 chromosomal DNA. Chromosomal DNA was isolated from each clone and analyzed by Southern hybridization. DNA sample from each clone was digested using various restriction enzymes. ^{32}P-DNA probes were prepared using ORF3, ORF4, ORF6 and ORF1, 2 and 5 PCR products. The observed and expected fragment sizes in Southern hybridization, calculated for all four ORFs after insertion-duplication mutation are summarized in Table 5. The standard curves for the correlation of fragment size and electrophoretic migration for each fragment are shown in Fig. 15-18.

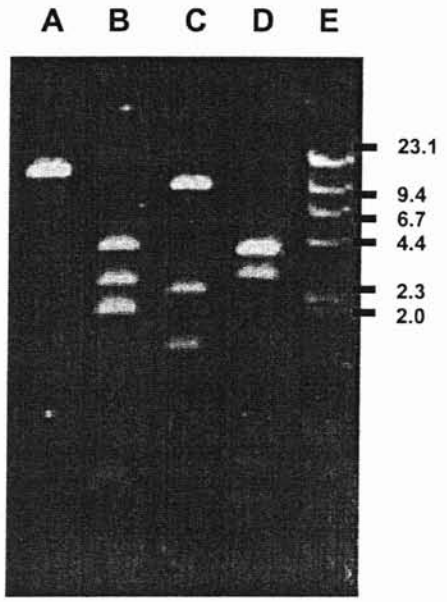

Figure 13. Molecular size markers prepared using pVJ15.On agarose gel pVJ15 cut with *Bam*HI (lane A), *Eco*RI (lane B), *Bgl*II (lane C), and *Hind*III (lane D) are shown. The molecular size marker displayed on lane E is *Hind*III cut λ- phage DNA ladder.

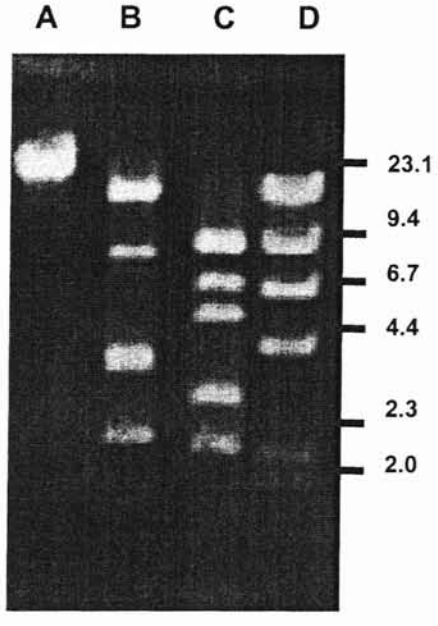

Figure 14. Molecular size markers prepared using pVJ18. On agarose gel pVJ18 cut with *Kpn*I (lane A), *Bgl*II (lane B), and *Eco*RI (lane C) are shown. The molecular size marker displayed on lane D is *Hind*III cut λ-phage DNA ladder.

Table 5

Expected and Observed DNA fragments After Southern Analysis

Mutant Strains	Observed Fragment Sizes (kb)	Expected Fragment Sizes (kb)	
		Right Orientation	Wrong Orientation
ORF3 (SP1501)	20, 16.5, 6.3	10.4 and a large one	4.1 and a large one
ORF4 (SP1502)	21	2.3 and 10	3.7 and 8.6
ORF1,2&5 (SP1504)	4.24 and 3.5	3.5 and 8.8	2.5 and a large one
ORF6 (SP1503)	12 and 6	3.8 and 13.3	7 and 10

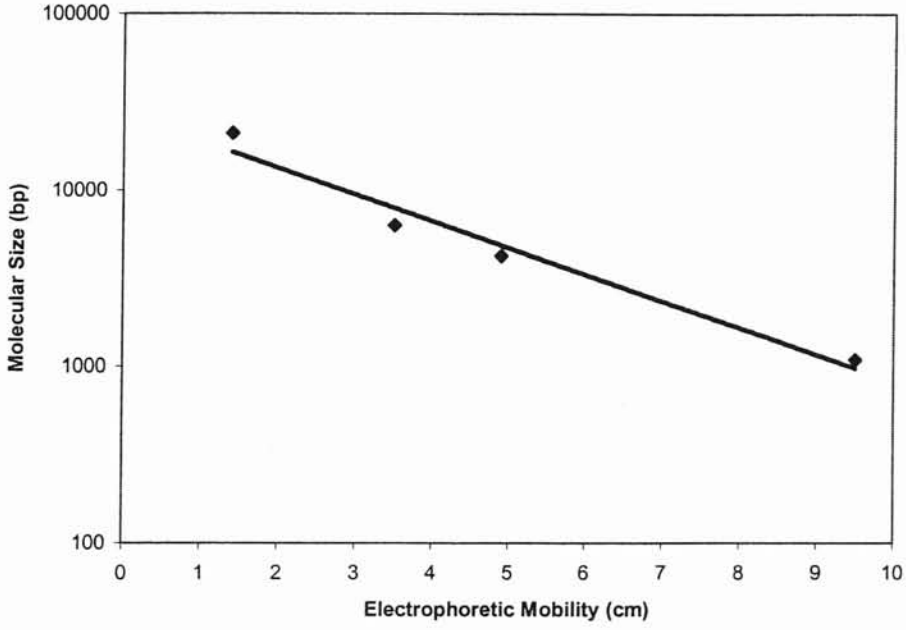

Figure 15. The standard curve for the correlation of fragment size and electrophoretic migration for ORF3 mutants. Putative ORF3 insertion-duplication mutants (SP1501-1 to SP1501-6) were analyzed by Southern hybridization. The sizes of DNA hybridization bands were calculated using the molecular size standards.

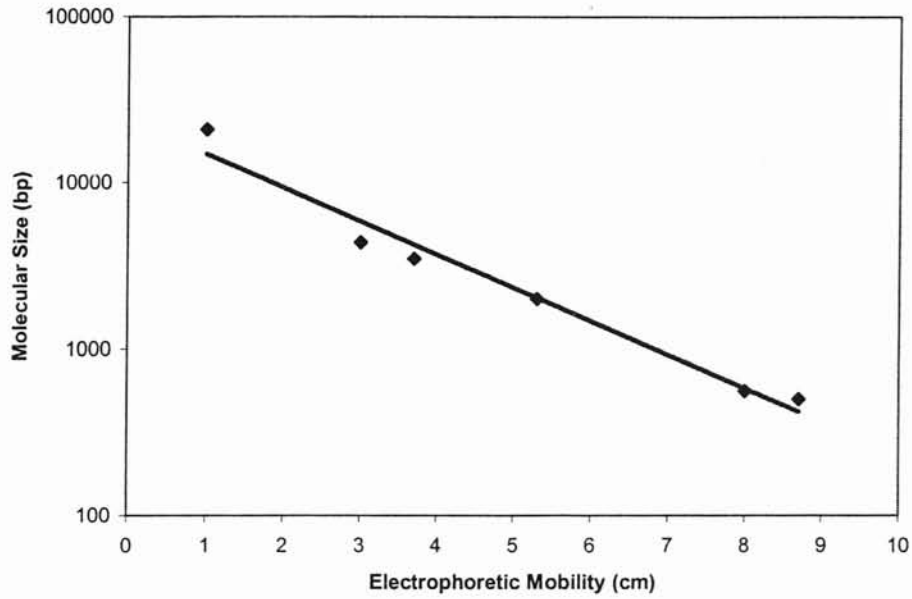

Figure 16. The standard curve for the correlation of fragment size and electrophoretic migration for ORF4 mutants. Putative ORF4 insertion-duplication mutants (SP1502-1 to SP1502-5) were analyzed by Southern hybridization. The sizes of DNA hybridization bands were calculated using the molecular size standards.

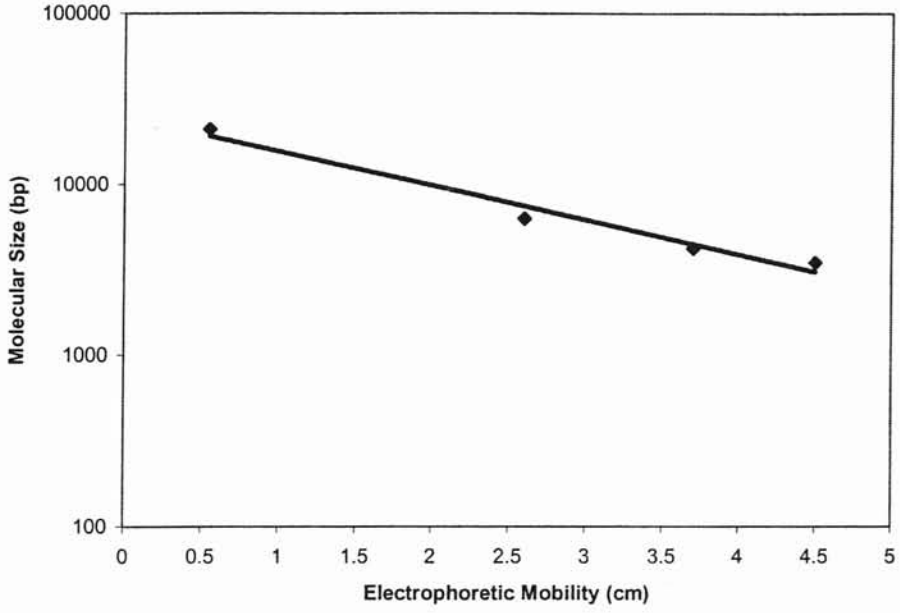

Figure 17. The standard curve for the correlation of fragment size and electrophoretic migration for ORF1, 2 and 5 mutants. Putative ORF1,2 and 5 insertion-duplication mutants (SP1504-1 to SP1504-4) were analyzed by Southern hybridization. The sizes of DNA hybridization bands were calculated using the molecular size standards.

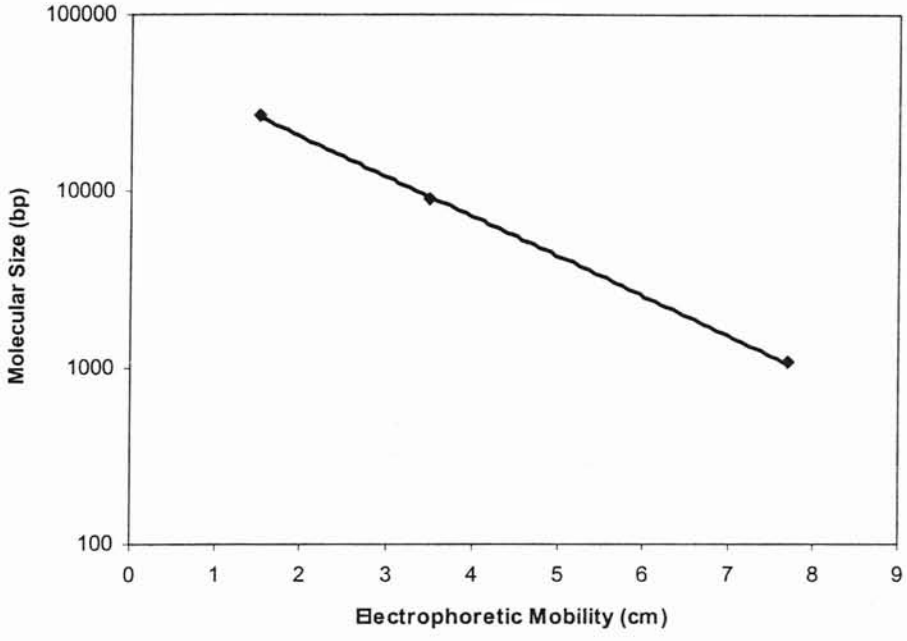

Figure 18. The standard curve for the correlation of fragment size and electrophoretic migration for ORF6 mutants. Putative ORF6 insertion-duplication mutants (SP1503-1 to SP1503-6) were analyzed by Southern hybridization. The sizes of DNA hybridization bands were calculated using the molecular size standards.

Analysis of ORF3 Using Insertion-Duplication Mutagenesis

The expected fragment sizes after hybridization of ORF3 with the chromosomal DNA from ORF3 insertion-duplication mutants were calculated using Tn*5252* restriction endonuclease map (35,77). After Southern hybridization we expected to see a 10.4 kb and a larger fragment if pEVP3[*Sma*I]::ORF3 insert was in the same orientation as the promoterless *lacZ* gene. This orientation was designated as the right orientation. A 4.1 kb and a larger fragment were expected if the insert is in the reverse orientation meaning *lacZ* gene and ORF3 were in the opposite direction. ORF3 autoradiogram results displayed two different band patterns. One of the patterns is possibly representing the insertion in the reverse orientation in SP1501-2, SP1501-4, SP1501-5 and SP1501-6 and the other pattern may be representing insertion in the right orientation in SP1501-1 and SP1501-3 (Fig. 19). However, the fragment sizes according to our results don't match with the expected results. The sizes of our observed fragments are 20 kb, 16.5 kb and 6.3 kb. This result may be explained by different methylation mechanisms in *E. coli* and *S. pneumoniae*. It is possible to see different restriction band patterns as a result of differences in methylation. Another reason may be multiple insertions of pEVP3[*Sma*I]::ORF3 into Tn5252. In addition, some sequences on Tn*5252* may be deleted upon insertion of pEVP3[*Sma*I]::ORF3. A similar result was observed when pVA891 was inserted into Tn*5252* (36).

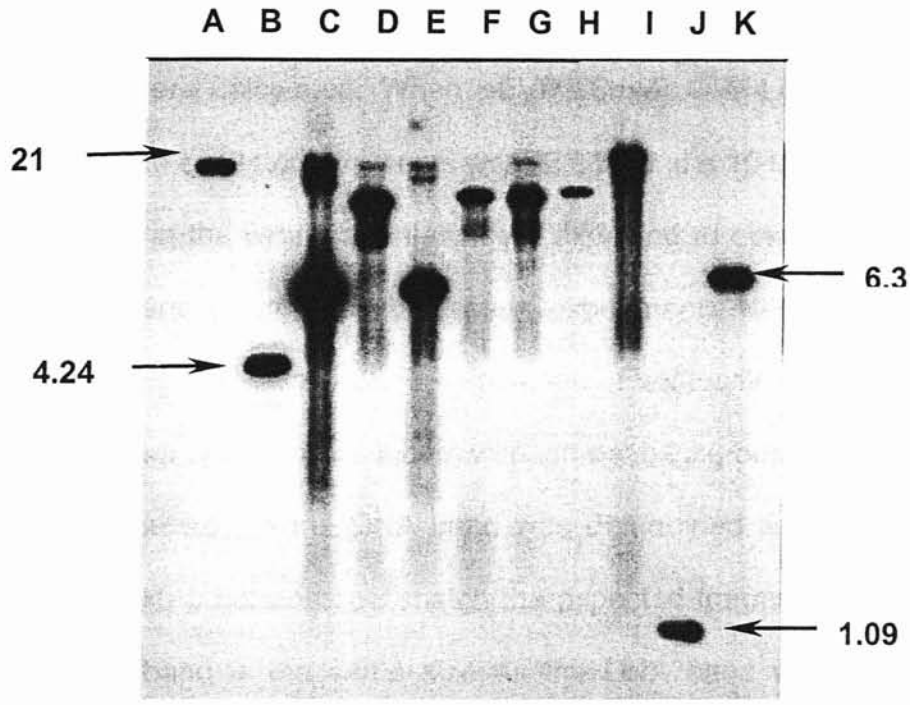

Figure 19. Analysis of ORF3 using insertion-duplication mutagenesis. Autoradiogram showing Southern hybridization of ^{32}P-labelled ORF3 to *Bam*HI cut chromosomal DNA from putative insertion-duplication mutants SP1501-1 (lane C), SP1501-2 (lane D), SP1501-3 (lane E), SP1501-4 (lane F), SP1501-5 (lane G), SP1501-6 (lane H) and SP1403 (lane I) as control. The molecular size markers, pVJ15 cut with *Bam*HI (lane A), *Bgl*II (lane B), *Eco*RI (lane J) and *Hind*III (lane K), are also shown.

Analysis of ORF4 Using Insertion-Duplication Mutagenesis

After Southern hybridization the expected fragment sizes for putative ORF4 mutants were calculated. When pEVP3[*Sma*I]::ORF4 insert was in the right orientation the expected fragments were 2.3 kb and a 10 kb fragment. When the insertion was in the wrong orientation we expected to observe a 3.7 kb and an 8.6 kb fragment. Southern hybridization experiments were repeated three times to analyze pEVP3[*Sma*I]::ORF4 insertion. Eventually, we were able to observe signals for the sample along with some background signal. On the autoradiogram, the size of the DNA band was determined as 21 kb (Fig. 20). However, the 21 kb band does not match the expected fragment sizes. On the other hand, this band is the same size as the DNA band we observe when SP1403 DNA was used as control. It is possible that pEVP3[*Sma*I]::ORF4 was not inserted into Tn*5252* in SP1403.

Analysis of ORF1, 2 and 5 Using Insertion-Duplication Mutagenesis

After hybridization, the expected DNA band sizes were calculated considering the insertion in both orientations. The sizes of expected DNA bands were a 3.5 kb and an 8.8 kb fragment for the right orientation. For the wrong orientation the predicted fragment sizes were 2.5 kb and a large fragment. On the autoradiogram, as predicted, a 2.5 kb hybridization band was observed. However, we observed a 3.5 and a 4.24 kb band instead of a large fragment

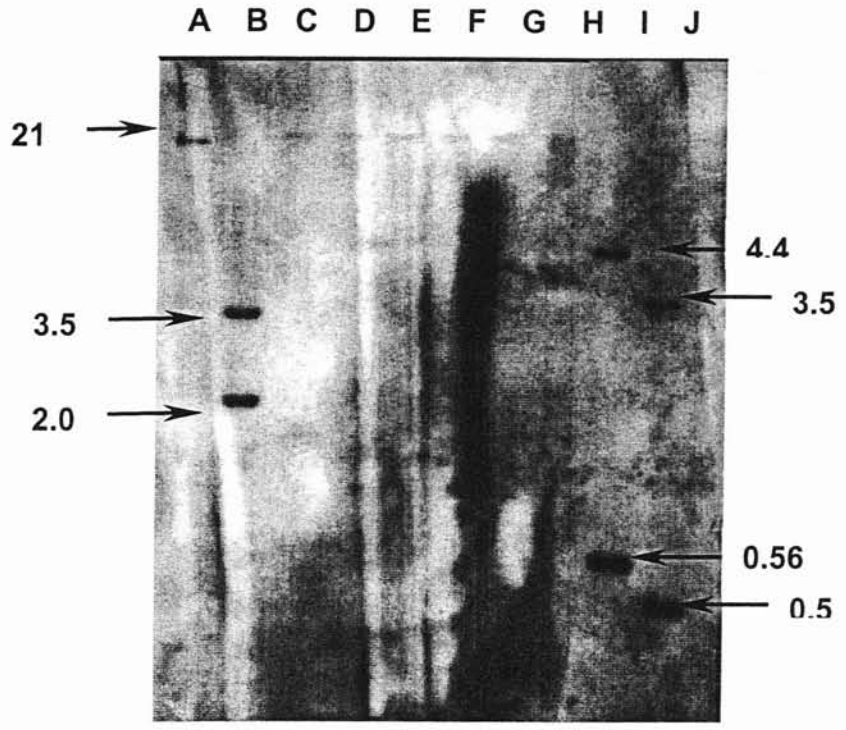

Figure 20. Analysis of ORF4 using insertion-duplication mutagenesis. Autoradiogram showing Southern hybridization of ^{32}P-labelled ORF4 to *Kpn*I cut chromosomal DNA from SP1502-1 (lane C), SP1502-2 (lane D), SP1502-3 (lane E), SP1502-4 (lane F), SP1502-5 (lane G), and SP1403 (lane H) as control. The molecular size markers, pVJ15 cut with *Bam*HI (lane A), *Bgl*II (lane B), *Eco*RI (lane I) and *Hind*III (lane J), are also shown.

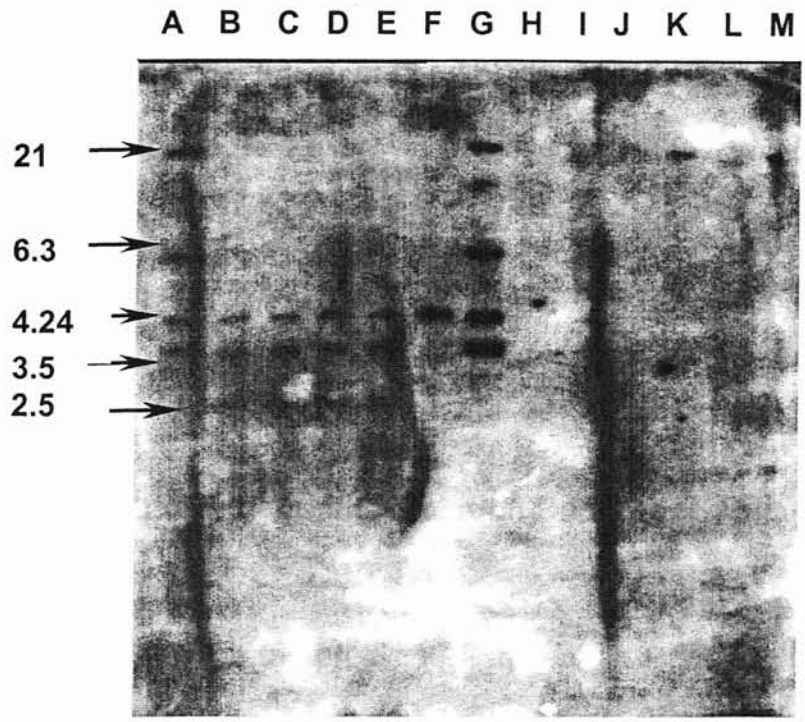

Figure 21. Analysis of ORF1, 2 and 5 using insertion-duplication mutagenesis. Autoradiogram showing Southern hybridization of ^{32}P-labelled ORF1,2 and 5 to *Bgl*II cut chromosomal DNA from SP1504-1 (lane B), SP1504-2 (lane C), SP1504-3 (lane D), SP1504-4 (lane E), SP1403 (lane F) as control, and to *Bam*HI cut SP1401 (lane H), SP1402 (lane J) and SP1403 (lane L), and to *Kpn*I cut SP1401 (lane I), SP1402 (lane K) and SP1403 (lane M). The molecular size markers, pVJ15 cut with *Bam*HI, *Bgl*II, *Eco*RI and *Hind*III (lanes A and G), are also shown.

(Fig. 21). This result may be due to the presence of more than two *Bgl*II sites on pEVP3. However, the restriction map of pEVP3 shows that there are only two *Bgl*II sites on this plasmid. Since there is a 2.5 kb observed fragment, which is the size of the expected fragment (2.5 kb), we suggest that the insert may be in the reverse orientation. However, we observed two band at 3.5 kb and 4.24 kb instead of a large DNA fragment. This result may be due to different methylation mechanisms in *E. coli* and *S. pneumoniae* and it may result in different restriction sites.

Analysis of ORF6 Using Insertion-Duplication Mutagenesis

After hybridization the predicted fragment sizes when the insert is in the right orientation was an 3.8 kb and a 13.3 kb fragment and we expected a 7 kb and a 10 kb fragment for the wrong orientation. After Southern hybridization we observe only one major band at 12 kb in the ORF6 mutant strains. On the other hand, only one of the ORF6 mutant strains, SP1503-2, had an additional faint signal at 6 kb (Fig. 22). Since the control DNA, isolated from SP1403, doesn't have any insertion, it is suspected that in five ORF6 strains pEVP3[*Sma*I]::ORF6 was not inserted into their chromosomal DNA. On the contrary, the presence of a second signal at 6kb and significantly high β-galactosidase activity of SP1503-2 ORF6 mutant may be an indication of pEVP3 [*Sma*I]::ORF6 insertion into the chromosome. This result was further investigated by analyzing inserted DNA

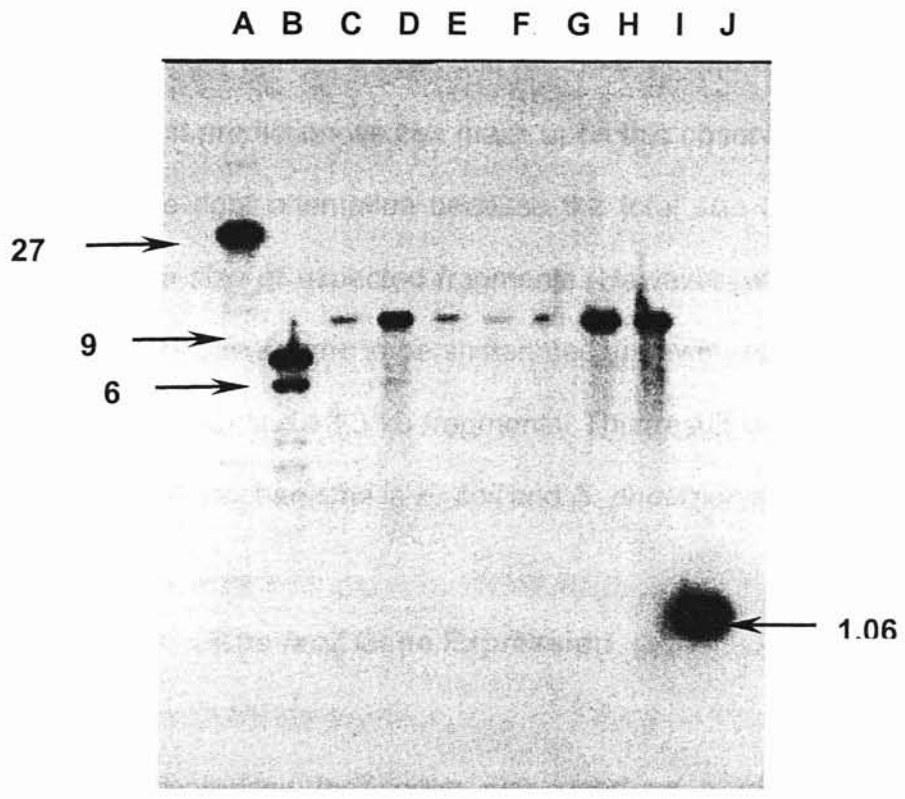

Figure 22. Analysis of ORF6 using insertion-duplication mutagenesis. Autoradiogram showing Southern hybridization of ^{32}P-labelled ORF6 to *Bgl*II cut chromosomal DNA from SP1503-1 (lane C), SP1503-2 (lane D), SP1503-3 (lane E), SP1503-4 (lane F), SP1503-5 (lane G), SP1503-6 (lane H) and SP1403 (lane I) as control. The molecular size markers, pVJ18 cut with *Kpn*I (lane A), *Bgl*II (lane B) and *Eco*RI (lane J), are also shown.

using restriction digestion analysis (Fig. 24, 26-28). These results will be shown later in this section.

When we sum up the observed and expected fragment sizes, for the observed one we calculate an 18 kb DNA and for the expected one we calculate a 17.1 kb DNA. The first prediction we can make upon this observation is that the insert is actually in the right orientation because the total size of the observed fragments is nearly the size of expected fragments. However, when the DNA is cut the observed *Bam*HI site seems to be shifted because we get 6 kb and 12 kb fragments instead of 3.8 kb and 13.3 kb fragments. This result is possibly due to variations in methylation mechanisms in *E. coli* and *S. pneumoniae*.

The *lacZ* Gene Expression

Previously, promoterless *lacZ* gene was used as a reporter gene to analyze the functions and characteristics of several genes, such as competence specific proteins (41). In this study, we sought to identify the constitutively expressed genes in Tn*5252* using *lacZ* as a reporter gene.

Several samples were selected based on their DNA band patterns observed in the autoradiograms. The *lacZ* gene expression was measured using ONPG as the chromogenic substrate. β-galactosidase produced by each sample was calculated and the enzyme activities are shown in Miller units in Table 6. *S. pneumoniae* strain SP1000 was used as a positive control since it contains a wild type *lacZ* gene. On the other hand, we measured the *lacZ* expression of SP1403

(β-gal$^-$) as our negative control. The results showed that the ORF3 containing SP1403 mutants, SP1501-1 and SP1501-3 had significantly high β-galactosidase activity (Table 6). Both mutant strains had similar DNA hybridization pattern. This observation implies that *lacZ* was inserted in the right orientation so that *lacZ* gene is expressed under the control of the ORF3 promoter.

SP1501-2, SP1501-4, SP1501-5 and SP1501-6 shared the same band pattern. These mutants have either no enzyme activity or very low activity as compared to other two mutants, SP1501-1 and SP1501-3. These mutants have *lacZ* gene inserted in the reverse orientation downstream of ORF3. On the contrary *lacZ* gene is inserted in the right orientation downstream of ORF7 in the same mutants. ORF7 was found to be a unique gene after database analysis. Also, ORF7 located on the left terminal of Tn5252 was shown to express a protein that is possibly involved in transfer of the element (70). Although the insertion of *lacZ* gene is in the right orientation downstream of ORF7, the lack of β-galactosidase activity in these mutants implies that ORF7 is possibly an inducible gene.

On the other hand, mutant strain SP1503-2 was the only ORF6 mutant that had significantly high β-galactosidase activity. Also, it is the only ORF6 mutant that has an extra 6 kb band other than the common 12 kb band. It is possible that the unique DNA band pattern and high β-galactosidase activity of SP1503-2 as compared to other ORF6 mutants may be an indication of insertion in the right orientation.

Table 6

β-galactosidase activity in mutants with fused *E.coli LacZ* gene to ORF3, ORF7, ORF4, ORF1, 2 and 5, and ORF6 in Miller units

Mutant Strains		β-galactosidase Activity (M.U.)
ORF3 Mutants	SP1501-1	17.99
	SP1501-3	15.67
ORF7 Mutants	SP1501-2	0.11
	SP1501-4	0.18
	SP1501-5	0.00
	SP1501-6	0.14
ORF4 Mutants	SP1502-1	0.00
	SP1502-2	0.00
	SP1502-3	0.00
	SP1502-4	0.00
	SP1502-5	0.00
ORF1,2&5 Mutants	SP1504-1	0.00
	SP1504-2	0.00
	SP1504-3	0.00
	SP1504-4	0.00
ORF6 Mutants	SP1503-1	0.02
	SP1503-2	11.59
	SP1503-3	0.19
	SP1503-4	0.17
	SP1503-5	0.44
	SP1503-6	0.10
LacZ-erm Mutants	SP1401	0.30
	SP1402	0.08
Positive Control	SP1000	25.21

We observed similar band patterns for all ORF1,2 and 5 mutants. These mutants had no detectable β-galactosidase activity (Table 6) possibly due to insertion of *lacZ* gene in the reverse orientation. Recently, it has been proposed that expression of integrase and excisionase are induced by some signals possibly required for mating (44, 45, 46, 59). If the insertion is in the right orientation we expect that there would be no detectable β-galactosidase activity unless integrase and excisionase genes are induced. ORF1,2 and 5 mutants will be further analyzed by restriction enzyme analysis to find the orientation of *lacZ* insertion.

Pneumoccoccus Transformation

The goal of this experiment was to obtain additional evidence to show that pEVP3 insertion was within the conjugative transposon Tn*5252*. For this experiment chromosomal DNA from insertion duplication mutants was used to transform pneumococcus strains SP1403 (ΩTn*5252*) and wild type strain R×1 that lacks the element. If the pEVP3 plasmid had inserted into Tn*5252* in the insertion-duplication mutants, then the transformation of the plasmid marker, Cm^r ,of SP1403 should be more efficient than transfromation of R×1. If the plasmid hadn't been inserted into the element, then SP1403 and wild-type R×1 should be transformed with equal efficiency.

Transformation results for SP1403 and R×1 are shown in Table 7. None of the chromosomal DNAs from the insertion-duplication mutants yielded R×1

transformants. However, 1500 Nov^r transformants arose when DP1617 DNA was used as control indicating that the recipient cells were competent. On the other hand, we had several fold more SP1403 Cm^r transformants for each insertion duplication mutant DNA except for ORF4 mutant strain. These results demonstrate that putative ORF3, ORF6 and ORF1,2 and 5 mutants carry pEVP3 DNA inserted in Tn*5252*.

The number of Cm^r transformants resulting from using SP1501-1 DNA was five times greater than from using SP1501-2 DNA as donors. This result may be due to insertion in different orientations in these mutants or DNA concentration and purity. In addition, β-galactosidase activity of SP1501-1 is significantly higher than the activity of SP1501-2. In any event, these results strongly demonstrate that *lacZ* was inserted within Tn*5252* in both mutants. However, the inserted *lacZ* DNA is in the reverse orientation in SP1501-2 mutant. Similarly, in SP1503-2, one of the ORF6 mutants, the *lacZ* was inserted within Tn*5252* as high number of Cm^r transformants of SP1403 resulted when SP1503-2 DNA was used as donor DNA (Table 7).

Analysis of Self-ligated Plasmid DNA Isolated from Putative Mutants

In order to further confirm that pEVP3 has been inserted into Tn*5252* in the mutant chromosomal DNA and in appropriate orientation, we transformed *E. coli* DH5α and XL1 Blue strains with digested chromosomal DNA from mutant

Table 7

Rx1 and SP1403 transformation results using donor DNA from mutant strains.

Donor	Cm^r Transformants	
	Rx1	SP1403
SP1501-1	0	580
SP1501-2	0	194
SP1502-4	0	0
SP1504-4	0	4
SP1503-2	0	764
SP1402	0	0
	Nov^r Transformants	
DP1617	1500	710

strains, SP1501-1, SP1501-2, and SP1503-2. The strategy is to digest the chromosomal DNAs of the insertion-duplication mutants at sites flanking the inserted vector, ligate the molecules to themselves, and use it as donor DNA to transform *E. coli*. As pEVP3 carry an *E. coli* origin of replication, the resulting recombinant plasmids would yield information on the orientation. *Kpn*I digested chromosomal DNAs from ORF3, ORF4 and ORF1, 2 and 5 mutants were self-ligated and used to transform *E. coli* host strain DH5α. Also, another *E. coli* host, XL1 Blue, was transformed with the digested and self-ligated DNA from ORF6 mutant. Previously, it was shown that DNA-cytosine methyltransferase (ORF6) may be toxic for some strains of *E. coli*. Therefore, we decided to use *E. coli* XL1 Blue as host strain. We isolated several Cm^r *E. coli* DH5α and XL1 Blue transfromants. We were able to extract plasmid DNA from *E. coli* DH5α and XL1 Blue transformants containing the ligated DNA from ORF3 (SP1501-1 and SP1501-2), and ORF6 (SP1503-2) mutants (Fig. 23). The isolated self-ligated plasmids were named as pEB2, pEB3 and pEB4.

We performed a restriction enzyme analysis to determine the orientation of pEVP3 inserted in these strains within Tn*5252* and to check whether there are any deletions. As expected the plasmids, pEB2 and pEB3, generated from SP1501-1 and SP1502-2 following *Kpn*I digestion and self-ligation, were about 7 kb in size. This result implied that pEVP3 was inserted within Tn*5252* resulting in creating an insertion-duplication mutation precisely at the site of ORF3. On the other hand, pEB4, the self-ligated plasmid DNA of SP1503-2 mutant strain was also digested with *Kpn*I. We expected to observe a 7 kb fragment and as

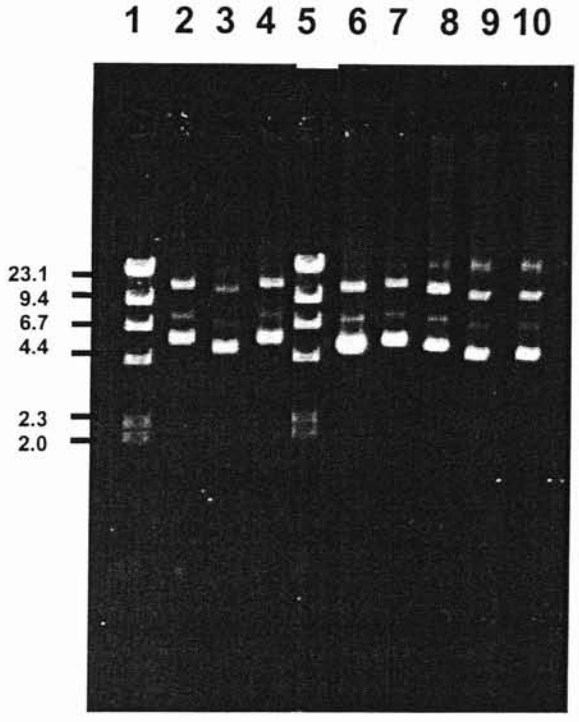

Figure 23. Analysis of the self-ligated plasmid DNA from SP1501-1, SP1501-2 and SP1503-2. The molecular weight standard, *Hind*III cut λ-phage DNA ladder, is shown on lanes 1 and 5. Recombinant plasmid isolated from SP1501-1, named pEB2, is illustrated on lanes 2, 3, and 4. The plasmid isolated from SP1501-2, pEB3, is shown on lanes 6, 7 and 8. The plasmid isolated from SP1503-2, named as pEB4, and pEVP3 are displayed on lanes 9 and 10.

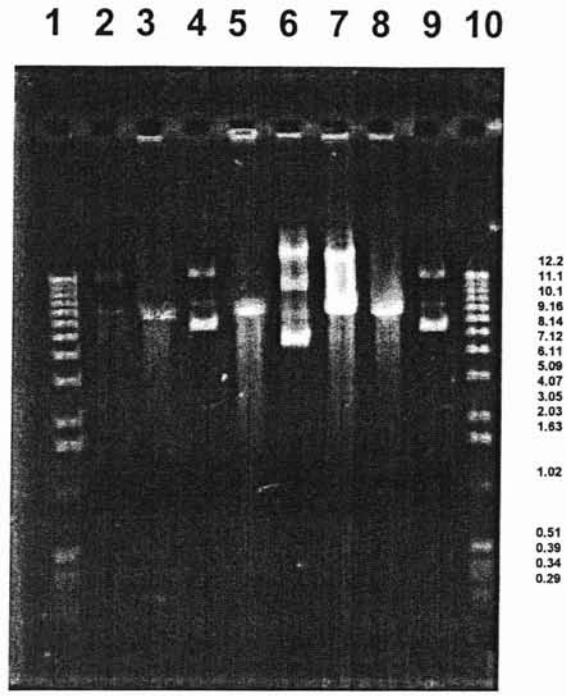

Figure 24. Restriction enzyme analysis of pEB2, pEB3 and pEB4 insertion plasmids. The molecular weight standard, 1 kb DNA ladder, is shown on lanes 1 and 10. Plasmids, pEB2, pEB3, pEB4, and pEVP3 are shown on lanes 2, 4, 6 and 9. *Kpn*I cut plasmids, pEB2, pEB3, pEB4 and pEVP3 are illustrated on lanes 3, 5, 7, and 8.

expected we obtained a 7 kb band after *Kpn*I digestion (Fig. 24). This result indicated that ORF6 was a part of pEVP3::ORF6 plasmid and the resulting ORF6 insertion-duplication mutants have pEVP3::ORF6 recombinant plasmid inserted within Tn*5252*.

In order to further extend the confirmation that pEVP3::ORF chimeric DNA molecule has been inserted into Tn*5252* at the corresponding ORFs, we digested chromosomal DNA from ORF3, ORF1, 2 and 5 and ORF6 mutants with more restriction enzymes to generate plasmids in *E. coli*. Chromosomal DNA from the insertion-duplication mutant SP1501-1 was cut with *Eco*RI and *Bam*HI. Similarly, chromosomal DNA from the insertion-duplication mutant SP1504-4 was digested with *Pst*I and *Bam*HI. Also, chromosomal DNA from the insertion-duplication mutant, 1503-2, was cut with *Eco*RI and *Bgl*II (Fig. 25). The resulting DNA molecules were ligated and used as donor DNAs to transform *E. coli* HB101 and XL1 Blue strains. We were able to obtain a few Cm^r *E. coli* HB101 and XL1 Blue transformants when digested DNAs from ORF3 and ORF6 mutants were used. Self-ligated plasmid DNA from each Cm^r transformant was extracted and analyzed using restriction enzyme analysis (Fig. 26).

When *Xba*I digested DNA from ORF1, 2 and 5 mutants was used as donor DNA we isolated a few Cm^r transformants and we could isolate a 7.3 kb plasmid DNA. The new plasmid pEB12 was analyzed by restriction enzyme analysis. The plasmid was linearized after cutting with *Xba*I, *Eco*RI, *Sph*I, *Sal*I and *Kpn*I . However, the plasmid was not linearized when it was cut with *Bam*HI

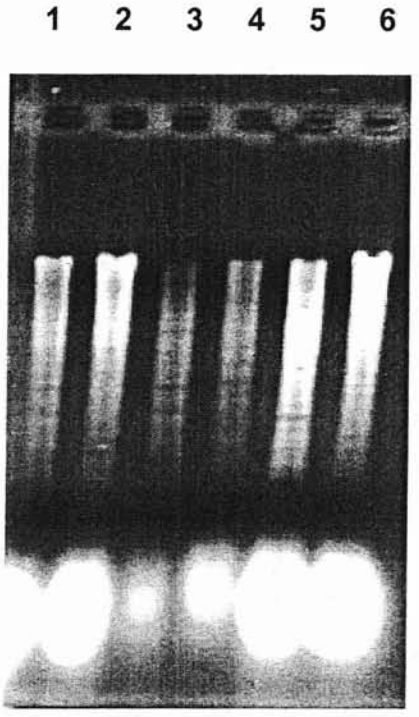

Figure 25. Digested Chromosomal DNA from SP1501-1, SP1503-2 and SP1504-4. SP1501-1 chromosomal DNA digested with *Eco*RI and *Bam*HI (lanes 1 and 2), SP1503-2 DNA cut with *Eco*RI and *Bgl*II (lanes 3 and 4), and SP1504-4 DNA digested with *Pst*I and *Bgl*II (lanes 5 and 6) are shown.

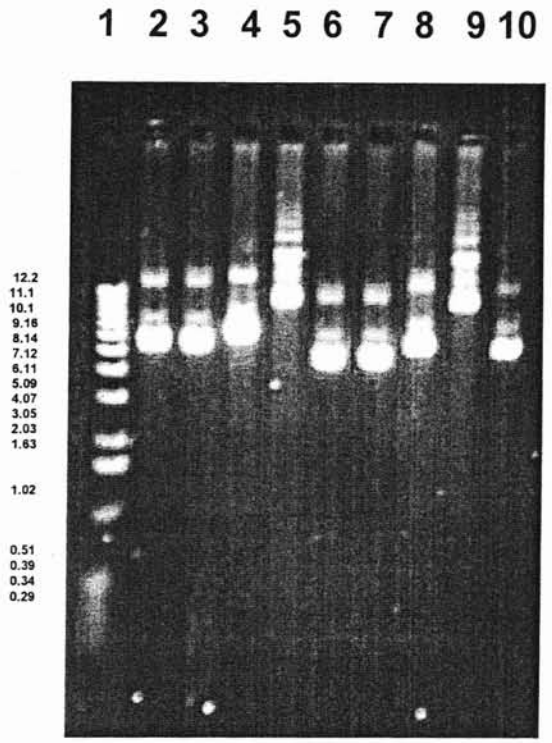

Figure 26. Analysis of self-ligated plasmids generated from SP1501-1 and SP1503-2. The molecular size marker, 1 kb DNA ladder (lane 1), pEB5, pEB5, pEB6, pEB7, pEB8, pEB8, pEB9, pEB10 and pEB11 (lanes 2, 3, 4, 5, 6, 7, 8, 9 and 10) are shown.

possibly due to the loss or modification of *Bam*HI restriction site. Furthermore, pEB12 was double digested with *Kpn*I and *Eco*RI and, we obtained DNA fragments at 1.2 kb and 6.2 kb. When the plasmid was cut with *Eco*RI and *Xba*I we observed a 0.2 kb and a 7 kb fragment as expected. The expected size of the plasmid inserted in the reverse orientation is 7.3kb. Our experiments indicated that pEVP3 is in the reverse orientation in SP1504-4.

The plasmids generated from ORF3 mutants SP1501-1 and SP1501-2 (pEB2, pEB3, pEB6, pEB7) are illustared in Fig. 27.The self-ligated plasmids, pEB6 and pEB7, were generated from SP1501-1 after EcoRI and BamHI digestion, respectively. In order to linearize pEB6 and pEB7 we digested these plasmids with *Eco*RI and *Bam*HI, respectively (Fig. 28). After digestion we observed an 8 kb pEB6 and a 10 kb pEB7 plasmids as expected. The only plasmid that did not meet with our predictions was the 9 kb *Bam*HI cut plasmid isolated from *E. coli* HB101 transformants. This plasmid was expected to be a 10 kb plasmid. Certain deletions may be observed as a result of insertions. Probably, a 1 kb deletion took place in Tn*5252* resulting in a 9 kb plasmid instead of a 10 kb one.

The plasmids generated from ORF6 mutant SP1503-2 (pEB4 and pEB10) are shown in Fig. 29.

We named the self-ligated plasmid isolated from Cm^r *E. coli* XL1 Blue transfromants as pEB10. When this plasmid was linearized with *Eco*RI we observed an 11 kb DNA band. The expected size of the plasmid was also 11 kb. Thus, this result is in agreement with our predictions.

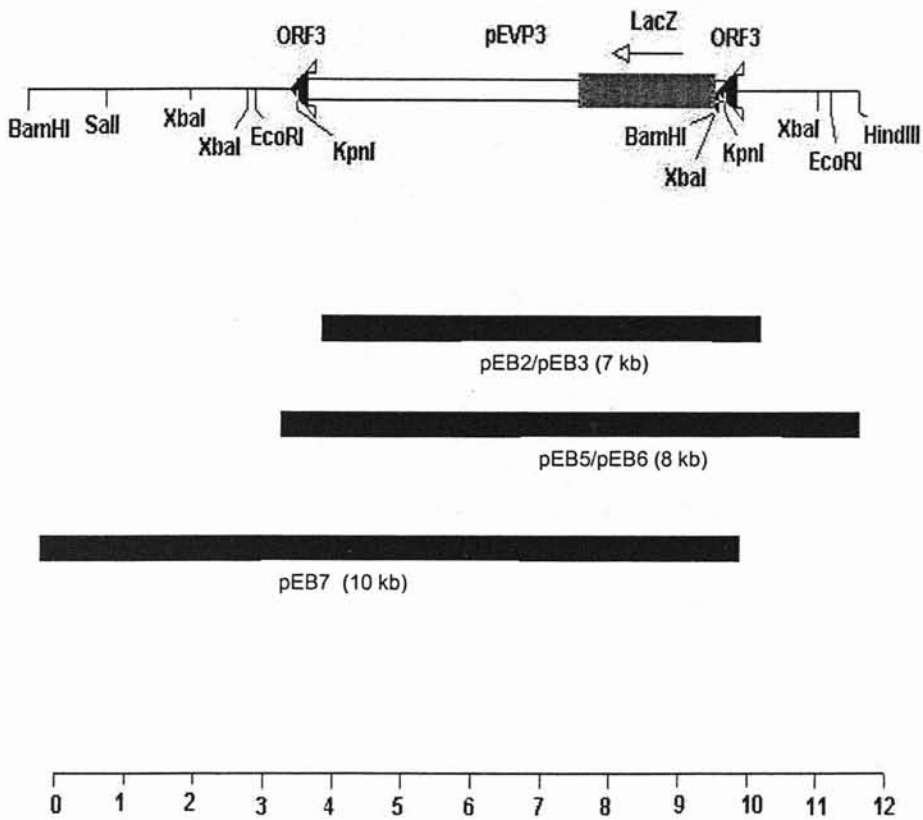

Figure 27. The plasmids generated from ORF3 mutants, SP1501-1 and SP1501-2, are shown.

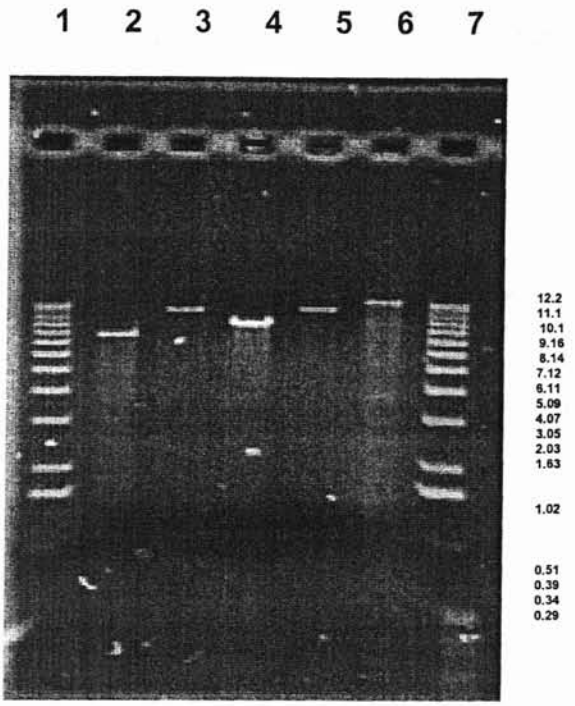

Figure 28. Analysis of linearized self-ligated plasmids from SP1501-1 and SP1503-2. The molecular size marker, 1 kb ladder, (lanes 1 and 7), *Eco*RI cut pEB6 (lane 2), *Bam*HI cut pEB7, pEB8 and pEB9 (lanes 3, 4 and 5) and *Bam*HI cut pEB10 (lane 6) are shown.

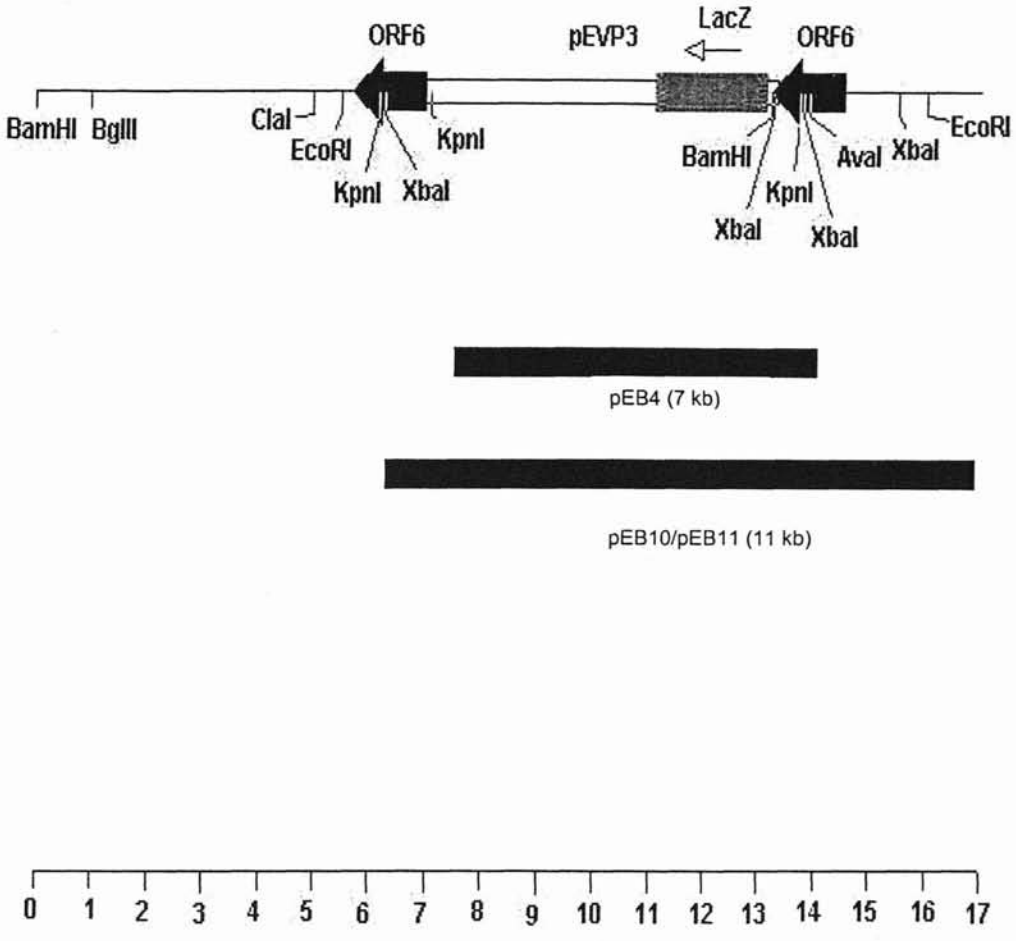

Figure 29. The plasmids generated from ORF6 mutant SP1503-2 (pEB4 and pEB10) are shown.

To confirm that the recombinant molecule is inserted in the expected ORFs in Tn*5252* we analyzed the isolated plasmid using restriction enzyme mapping. The 8 kb and 10 kb plasmids, pEB6 and pEB7, and 11 kb plasmid, pEB10 were digested with several restriction enzymes (Fig. 30 and 31). The expected and observed fragment sizes after each digestion is summarized in Table 8. We could observe the expected fragments for most of the digestions but a few of these fragments were unexpected. When 8 kb plasmid, pEB6, was cut with *Kpn*I we observed a 6.3 kb and a 1.5 kb fragment instead of the expected 6.3, 1.5 and 0.2 kb fragments. The 0.2 kb fragment is too small to be seen on 0.8% agarose gel. After digesting pEB6 with *Xba*I we observed 7 and 1 kb bands as expected.

We also analyzed pEB7 by cutting this 10 kb plasmid with several restriction enzymes (Table 8). When the plasmid was cut with *Kpn*I and *Eco*RI we observed a single 10 kb band as expected. The *Xba*I cut pEB7 plasmid gen erated two bands at 0.8 and 9.5 which are in agreement with our predictions. The pEB7 double digestion with BamHI/XbaI and BamHI/SalI also generated the expected bands.

Finally, we analyzed pEB10 with the enzymes *Eco*RI, *Xba*I, *Kpn*I and *Ava*I (Fig. 32). When the plasmid was cut with *Eco*RI we observed an 11 kb plasmid as expected. On the contrary, when the plasmid was cut with *Eco*RI and *Xba*I we observed that an expected 1 kb fragment was not generated. This result may be due to a deletion that took place within Tn5252 after the insertion of pEVP3. In addition, *Eco*RI and *Kpn*I double digested pEB10 generated 0.5, 2.9 and 7 kb

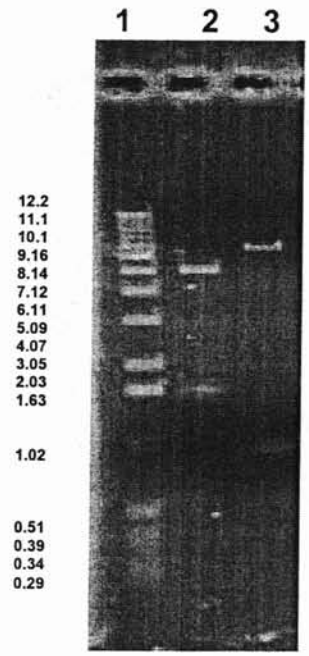

Figure 30. Restriction enzyme analysis of pEB6. The molecular size marker, 1 kb ladder (lane 1), and *Kpn*I and *Xba*I cut pEB6 (lanes 2 and 3), are shown.

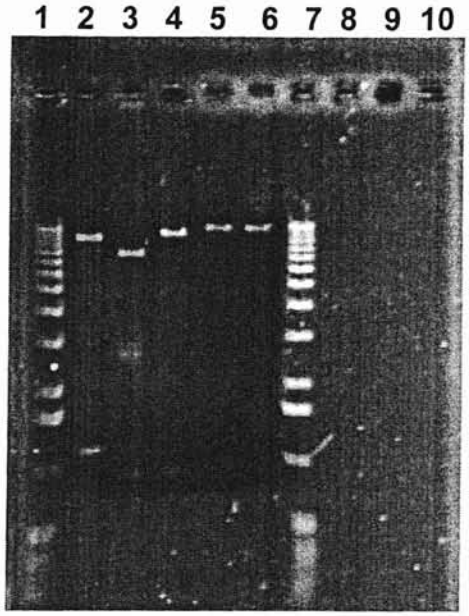

Figure 31. Restriction enzyme analysis of pEB7. The molecular size marker, 1 kb ladder (lanes 1 and 7), *Bam*HI and *Sal*I cut pEB7 (lane 2), *Bam*HI and *Xba*I cut pEB7 (lane 3); *Xba*I cut pEB7 (lanes 4); EcoRI cut pEB7 (lane 5) and *Kpn*I cut pEB7 (lane 6) are shown.

Table 8

Analysis of Self-ligated Plasmids by Restriction Digestion

Inserted Plasmids	Observed Fragment Sizes (kb)	Expected Fragment Sizes (kb)
SP1501-1 (ORF3)		
pEB6	8	8
pEB7	10	10
*Kpn*I cut pEB6	6.3 and 1.5	6.3, 1.5 and 0.2
*Xba*I cut pEB6	7 and 1	7 and 1
*Kpn*I cut pEB7	10	10
*Xba*I cut pEB7	0.8 and 9.5	0.8 and 9.2
*Eco*RI cut pEB7	10	10
*Bam*HI*Xba*I cut pEB7	0.8, 2.6 and 7	0.8, 2.3 and 7
*Bam*HI*Sal*I cut pEB7	1.3 and 9.5	1.1 and 9
SP1503-2 (ORF6)		
pEB10	11	11
*Kpn*I cut pEB10	0.5, 3 and 7	0.5, 3.3 and 6.8
*Xba*I cut pEB10	0.5, 1.6 and 7.5	0.5, 1.5, 1.8 and 6.8
*Eco*RI cut pEB10	11	11
*Eco*RI*Xba*I cut pEB10	0.5, 0.5, 1.6 and 7	0.5, 0.5,1, 1.8 and 6.8
*Eco*RI*Kpn*I cut pEB10	0.5, 2.9 and 7	0.5, 1, 2.3 and 6.8
*Ava*I*Eco*RI cut pEB10	2 and 9.5	2.2 and 8.4
SP1504-4 (ORF1,2 and 5)		
pEB12	7.3	7.3
*Eco*RI*Xba*I cut pEB12	0.1 and 7.2	0.2 and 7
*Eco*RI*Kpn*I cut pEB12	1.1 and 6.2	1.2 and 6.2

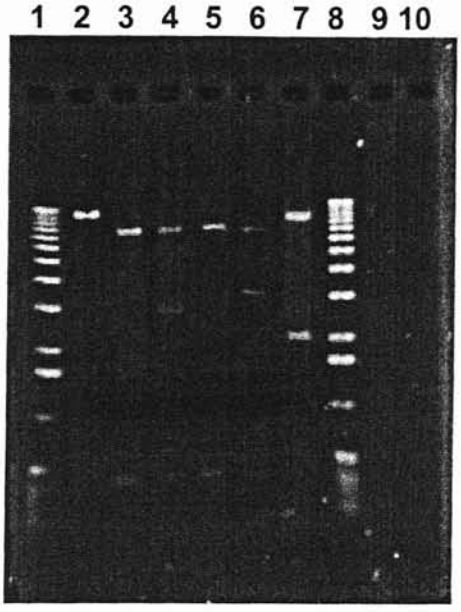

Figure 32. Restriction enzyme analysis of pEB10. The molecular size marker, 1 kb ladder (lanes 1 and 8), EcoRI cut pEB10 (lane 2), *Eco*RI and *Xba*I cut pEB10 (lane 3), *Eco*RI and *Kpn*I 10 (lane 4); *Xba*I cut pEB10 (lanes 5), *Kpn*I cut pEB10 (lane 6) and, *Eco*RI and *Ava*I cut pEB10 (lane 7) are shown.

instead of 0.5, 1, 2.3 and 6.8 kb bands. Similarly, *Xba*I cut pEB10 plasmid generated several fragments but an expected 1.5 kb fragment was not observed. These result may be explained by possible differences in methylation in *E. coli* and *S. pneumoniae*.

CHAPTER VI

DISCUSSION

During this study our goal was to create *lacZ* operon fusions with the regulator, relaxase, DNA-cytosine-methyltransferase, integrase and excisionase genes in Tn*5252* to understand their transcriptional patterns. We intended to identify constitutive and inducible genes in Tn*5252* by placing *lacZ* gene under the regulatory control of the promoter of the targetted gene.

First we focused on constructing a *lacZ* reporter system in *S. pneumoniae* using Tn*917* mutagenesis. We used several hosts strains such as, *E. coli* JM109 and C600, *S. pneumoniae* SP1000, and *E. faecalis* JH2-2. In addition, we utilized a variety of vector plasmids including pLSI, pVA838 and pAT29 to clone the Tn*917ΩlacZ* fragment of pTV53Ts. Our Tn*917* mutagensis results indicated that Tn*917* cannot be cloned in *S. pneumoniae*. There are no reports on cloning of Tn*917* in *S. pneumoniae*. Presumably, Tn*917* is not stable due to the absence of a host factor in *S. pneumoniae* (46). Furthermore, Tn*917* may be toxic for some *S. pneumoniae* strains which makes utilization of this element impossible in this host.

Several researchers have shown that Tn*917* is a powerful tool for mutagenesis in *B. subtilis*, *L. lactis S. mutans*, *S. aureus* and *S. pneumoniae* (25,21,27,33,72,91). In addition, cloning of chromosomal DNA adjacent to Tn*917* insertions after mutagenizing these hosts has been reported for *E. coli*. On thecontrary, there are no reports on cloning of Tn*917* DNA fragments in *E. coli*. The most likely explanation for not being able to clone Tn*917* in *E.coli* may be the presence of genes with strong promoters in Tn*917*. It is possible that, due to strong promoters, the vector is expressed excessively and therefore the replication of plasmid vectors will be no longer controlled. Strong terminator signals are required in a vector to eliminate this discrepancy (11,12). Another explanation for this result may be the lack of certain host factor(s) required for the stability of Tn*917* in *E. coli*.

As an alternative method, we employed insertion-duplication mutagenesis to analyze the expression of Tn*5252* genes by establishing a *lacZ* reporter system in *S. pneumoniae*. We utilized a variety of plasmid vectors to insert the *lacZ* gene under the control of the promoters of each targeted ORF using insertion-duplication mutation. The results of cloning of *lacZ-erm* fragment on pSJ126 indicate that this fragment can not be cloned on this plasmid. A possible explanation for this result may be the presence of a strong promoter on *lacZ-erm* fragment. This may result in plasmid instability due to excessive expression of certain genes. We used the same *lacZ-erm* fragment and ligated this fragment to PCR products of ORFs, to insert *lacZ* gene downstream Tn*5252* ORFs. We isolated two putative insertion-duplication mutants named SP1401 and SP1402.

The results of Southern hybridization, β-galactosidase assay and pneumococcus transformation experiments showed that *lacZ-erm* fragment was not inserted into Tn*5252* in these putative mutants. These experiments indicate that *lacZ-erm* fragment of pTV32Ts cannot be used to mutagenize Tn*5252* genes using insertion duplication mutagenesis.

Alternatively, we used the insertion plasmid vector pEVP3 to insert a promoterless *lacZ* gene downstream of ORF3, ORF4 and ORF6, ORF1, 2 and 5 by insertion-duplication mutagenesis. We created several putative insertion-duplication mutant strains. First, we analyzed the insertion of pEVP3 plasmid using Southern hybridization. ORF3 insertion-duplication mutants displayed two different band patterns on autoradiogram. However, we did not observe the expected hybridization bands. This result may be due to variations in methylation mechanisms in *E. coli* and *S. pneumoniae*. In addition, multiple insertions of pEVP3::ORF3 may have resulted in unexpected fragment sizes. Two ORF3 mutants, SP1501-1 and SP1501-3 sharing the same band pattern have high β-galactosidase activity whereas the other four mutants that share a different band pattern have no detectable β-galactosidase activity. In addition, R×1 and SP1403 transformation results show that when SP1501-1 DNA was used as donor the number of Cm^r SP1403 transformants several fold more than Rx1 transformants indicating that the inserted DNA is within Tn*5252*. Also, *E. coli* JM109 transformation using restriction digested chromosomal DNA from SP1501-1 shows that this mutant has pEVP3 inserted. The analysis of self-ligated plasmid isolated from SP1501-1 indicates that ORF3 has been ligated to

pEVP3 and has been inserted as pEVP3::ORF3 chimeric molecule. Additionally, to confirm that the insertion of pEVP3::ORF3 is at ORF3 in Tn*5252* we performed restriction enzyme analysis. The results after analysis of self-ligated plasmids isolated from ORF3 insertion-duplication mutant, SP1501-1, indicate that the insert was in the right orientation.

According to our results SP1501-1 is an insertion duplication mutant that carries a *lacZ* reporter gene downstream of ORF3. Additionally, we are able to detect high β-galactosidase activity. Therefore, we suggest that ORF3 is an active gene in normal cells. Previously, the homology between the repressor (ORF3) and *xre* repressor of *B. subtilis* phage PBSX has been shown. Xre repressor is a regulatory protein encoded by a gene that is expressed constitutively. We suggest that Tn5252 repressor gene (ORF3) is also expressed constitutively in *S. penumoniae* based on our experimental results.

Similarly, putative ORF4 insertion-duplication mutants were analyzed by Southern hybridization. On the autoradiogram we could only observe one type of band pattern which is the same as the control, SP1403. When we tested ORF4 mutants for their β-galactosidase activity, no enzyme activity was detected. In addition, we could not isolate any Cm^r transformants when SP1504-4 DNA was used to transform pneumococcus strains Rx1 and SP1403. These results suggested that pEVP3 was not inserted into Tn*5252* and therefore no ORF4 insertion-duplication mutant was created. On the other hand, we can still predict the expression of relaxase is possibly induced upon excision of the element or by other factors involved in transposition process. Since this gene is involved in site-

specific nicking of supercolied DNA during conjugal transfer its expression may only be essential during the transfer process.

Also, we employed Southern hybridization to analyze putative ORF1, 2 and 5 insertion-duplication mutants. On the autoradiogram all ORF1, 2 and 5 mutants displayed the same DNA hybridization band pattern. When we compared the expected and observed hybridization bands, we observed a 2.5 kb band as expected but we couldn't see a large band. The most likely explanation for this result may be the deletion of Tn*5252* sequences upon insertion of pEVP3. In addition, the consequences of different methylation mechanisms in *E. coli* and *S. pneumoniae* may result in considered to explain this result. Furthermore, all putative ORF 1, 2 and 5 insertion-duplication mutants had no detectable β-galactosidase activity. Additionally, restriction enzyme analysis of the self-ligated plasmid (pEB12) generated from ORF1, 2 and 5 mutant indicated that the insertion is in the reverse orientation. Further analysis of ORF1, 2 and 5 mutants by transforming Rx1 and SP1403 with chromosomal DNA from one of these mutants indicate that pEVP3 is possibly inserted into Tn5252.

Finally, all ORF6 insertion-duplication mutants displayed the same hybridization band pattern on autoradiogram, except SP1503-2. On the autoradiogram, this mutant had an extra weak signal at 6 kb. Also, we detected significantly high β-galactosidase activity for SP1503-2 whereas other five mutants had very low β-galactosidase activity. In addition, pnemococcus transformation results indicate pEVP3 is inserted into Tn*5252*. To investigate the site of pEVP3 insertion in Tn5252 we analyzed the self-ligated plasmids isolated

from mutant strain SP1503-2. Restriction enzyme analysis implied that pEVP3::ORF6 is inserted at ORF6 site in the right orientation. Furthermore, we suggest that ORF6 is always active in the cell and therefore it's expressed constitutively.

In addition, although the insertion of lacZ gene is in the right orientation as compared to ORF7 we detected no enzyme activity after *lacZ* gene fusion downstream of tra-related gene ORF7. Therefore, we propose that ORF7 is possibly an inducible gene.

ORF6 is not transfer-related (61). As it is not a *tra* related gene, it is not expected to be controlled by factors responsible for regulating genes involved in transposition. This expectation matches the experimental findings.

In, sum, it seems likely that the genes involved in transfer of the element are subject to negative regulation and induced only during mating conditions. Also, this study provides further evidence that ORF3 is more likely to be a negative regulator.

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VITA 2

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Thesis: IDENTIFICATION OF CONSTITUTIVELY VERSUS INDUCIBLY EXPRESSED GENES IN THE STREPTOCOCCAL CONJUGATIVE TRANSPOSON Tn*5252*

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